REGULATION OF SEX PHEROMONE PRODUCTION AND OVIPOSITION IN VIRGIN FEMALES OF NOCTUID MOTHS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

ACKNOWLEDGMENTS

I wish to express my appreciation to the Department of Entomology and Nematology and to the USDA Insect, Attractants, Behavior and Basic Biology Research Laboratory, particularly the Chemistry Group, for all the help and support I received during my doctoral research. Special thanks go to my advisor, Dr. Peter Teal, for providing me with a wonderful education. I also thank Dr. James Tumlinson for his guidance during my studies, Dr. James Nation, Dr. Jane Brockmann and Dr. Henry Aldrich for serving on my commitee, and Dr. Herbert Oberlander for reviewing my disseration and attending my defense.

I was fortunate to be able to work with several scientists who encouraged me and helped me a great deal. These include Dr. Ronald Nachman, Mr. Robert Heath, Dr. Nancy Epsky, Ms. Barbara Dueben and Dr. Peter Landolt. I owe a debt of gratitude to Dr. Julia Meredith for her help, advice and great moral support. I thank my long-suffering husband, Rob, for keeping me company during the overnight experiments and for his continuous support. Mr. Oscar Molina gave technical support and lent his artistic effort for drawings. And finally, I wish to thank my late, great friend, Dr. Scott R. Yocom, who allowed me to share in his beautiful life and his graceful death and who helped to teach me the most important lessons in life.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

REGULATION OF SEX PHEROMONE PRODUCTION AND OVIPOSITION IN VIRGIN FEMALES OF NOCTUID MOTHS

By

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December, 1995

Chairperson: P. E. A. Teal
Major Department: Entomology and Nematology

Several factors that influence pheromone production and oviposition in virgin females of the noctuid moths *Helicoverpa zea* (Boddie), *Heliothis virescens* (F.) and *Trichoplusia ni* (Hübner) were studied. Structure-activity studies were conducted using pheromonotropic assays with *H. zea*. Neuropeptides tested shared the C-terminal sequence FXPRL-amide (X=V, T, S or G). Studies showed that locustamyotropin-II and locustapyrokinin-II had a superagonistic action with respect to the pheromone biosynthesis activating neuropeptide (PBAN) of this moth. Other peptides showed various degrees of activity. Both the variable amino acid in the C-terminal pentamer and overall structure of the peptides were found to be important for bioactivity. Modification of locustamyotropin-II by N-terminal addition of 6-phenylhexanoic acid yielded an amphiphilic pseudopeptide. This compound was as active as PBAN in pheromonotropic assays, crossed the insect cuticle when applied topically in H_2O and lasted longer than PBAN. The design and

efficacy of the pseudopeptide represents development of a new delivery system for insect neuropeptides which could be applied to synthesis of peptidomimetics for other physiological processes.

Aging caused a decrease in pheromone production and increase in oviposition by virgin *H. zea* females. These physiological and behavioral changes were stimulated by pheromone biosynthesis suppression factor (PBSF). PBSF was isolated, partially purified and characterized as a highly polar weak acid from extracts of the bursa copulatrix. The presence of more than one *H. zea* female stimulated oviposition, but did not affect pheromone production. Similarly, presence of a *T. ni* or *H. virescens* female also induced oviposition by *H. zea*. Neither *T. ni* nor *H. virescens* was affected similarly.

The effect of presence of a host plant on pheromone production was studied using *T. ni*. Cotton induced a temporal shift and an increase in amount of pheromone released in spring. However, no such differences were evident at other times of the year.

Overall, the studies conducted have shed new light on the complex environmental and endogenous factors that interact to coordinate the reproductive and pheromone communications systems of these moths. The conclusions provide a clear basis for additional research.

CHAPTER 1
INTRODUCTION

Animals gather a tremendous amount of information about their environments through the senses of smell and taste. Chemicals used in the interactions between organisms and their surroundings are referred to as semiochemicals (Law and Regnier, 1971). Semiochemicals play a major role in information gathering and it is thought that chemical receptors evolved very early, and possibly, were the first sort of communication between organisms (Snodgrass, 1929; Haldane, 1955). Chemical communication is used by virtually all animals, and given this long evolutionary history, the use of semiochemicals has been adapted and specialized, particularly by the insects.

Like all animals, insects have to navigate in a world filled with chemical cues and be able to detect food, interact with other organisms and locate mates. Pheromones play a crucial role in mate-finding and sexual communication in many insects. Pheromones are defined as chemicals emitted and received by members of the same species (Karlson and Luscher, 1959). Sex pheromones, chemicals used for attracting mates, are well developed in the lepidopterans and are lipidic in nature (Teal and Tumlinson, 1992). Sex pheromones are highly volatile and males are able to detect very low concentrations of female-borne pheromones from great distances. Since the first characterization of the sex pheromone of the silkworm moth (Butenandt et al., 1959), more than 1600 other sex pheromones have been identified from different insect species (Roelofs, 1995). In order for insects to find potential mates of the same species, it is important for pheromones to be clear and precise signals. Much of this precision is due to the chemistry of sex pheromones. This chemistry

imparts a specificity to pheromones and members of each species are able to detect and respond to their own particular blend. Several factors enable lepidopteran species to utilize pheromone communication effectively, including the chemical uniqueness of the pheromone blend, the time that the pheromone is emitted and the time that the receiving insect is responsive to the pheromone. The following sections describe the chemical characteristics of the sex pheromones, with particular emphasis being given to the pheromones of the insects used for the studies presented here.

Sex Pheromone Chemistry

Most lepidopteran sex pheromones are similar in chemical structure and their synthesis is accomplished by the action of similar enzymatic pathways. However, the exact nature of the chemistry is very specific for each species, since minor changes in the structure of pheromone components of a particular blend can completely disrupt the response of the receiving individual. The similarity in lepidopteran sex pheromones, with minor differences between species, and a highly specific behavioral response to precise compounds, shows that the evolution of these pheromones has been highly conserved.

The majority of lepidopteran sex pheromones are derived from fatty acids. They are aliphatic compounds, varying in chain length, but are usually straight chains of 10, 12, 14, 16 or 18 carbons (Bjostad et al., 1987). The number, position and geometry of double bonds vary, and most functional groups are either acetates, aldehydes or alcohols. Many moth species share pheromones that are similar chemically. Specificity between species is achieved by producing a blend of compounds or by varying the ratio of the components. For example, approximately the same ratio of (Z)-11-hexadecenal, hexadecenal, (Z)-7-hexadecenal and (Z)-9-hexadecenal are used as pheromones by the noctuid moths, *Helicoverpa zea* (Klun et al., 1980b) and *Heliothis virescens* (Klun et al.,

1980a) However, the blend of *H. virescens* contains 14-carbon aldehydes, whereas *H. zea* does not, and this difference in blend imparts pre-mating reproductive isolation between the two species (Klun et al., 1979).

In some cases, different species can share a pheromone blend that is chemically identical while occupying the same geographic range. Pre-mating reproductive isolation can be achieved in this instance by the production of pheromone in temporally discrete periods. These distinct periods have been demonstrated for three species of noctuid moths belonging to the genus *Euxoa* in which the females produce a pheromone that is cross-attractive to males of all three species (Byers et al., 1981). Each species, however, produces pheromone during a tightly regulated time period and thereby achieves reproductive isolation (Teal et al., 1978).

Biosynthesis of Sex Pheromones

The pheromone glands of *H. zea, H. virescens* (Jefferson et al., 1968) and *Trichoplusia ni* females (Jefferson et al., 1966) are derived from modified epidermis located on the abdomen between the 8th and 9th segments. The cells of the epithelium of this intersegmental membrane vary in shape from cuboidal to columnar. In *H. zea*, the gland is a ring of uniform tissue around the abdomen (Aubrey et al., 1983). *H. virescens* has a ventrolateral gland (Teal et al., 1983), and the gland of *T. ni* is a dorsal modification of the intersegmental membrane (Percy-Cunningham and MacDonald, 1987). The cells contain lipid spheres, and the mechanism of transport of pheromone from the epidermal cells to the surface of the gland is thought to result from diffusion of material through the pore canals of the cuticle overlaying the gland (Percy-Cunningham and MacDonald, 1987).

Some moths store large amounts of pheromone in the gland, and other moths have very little present at times when pheromone is not released. The cabbage looper, *T. ni*, has

as much as 1 μg of pheromone present during the day, when pheromone is not released (Berger, 1966). However, the other two species used for this study, *H. zea* and *H. virescens*, have almost no pheromone present in the gland when pheromone is not being released during the photophase (Klun et al., 1980b, 1980b).

The fatty acyl precursors of pheromones are generated by fatty acid synthesis. These precursors then undergo a series of chain-shortening or elongation, desaturation and addition of functional moieties by reduction, oxidation or acetylation (Bjostad et al, 1987; Morse and Meighan, 1987; Tumlinson and Teal, 1987). Desaturation often occurs by the action of an enzyme unique to Lepidoptera, Δ11-desaturase (Bjostad, 1983). These biosynthetic steps produce a wide variety of alcohols, aldehydes and acetates and when combined into different blends, an almost unlimited number of pheromones are possible (Roelofs, 1995). The chemistry of these simple compounds is a major contributor to the reproductive isolation of most moth species.

Sex Pheromone of *Trichoplusia ni*

Shortly after bombykol was identified as the pheromone of the silkworm moth, *Bombyx mori* (Butenandt, 1959), researchers began identifying the sex pheromones of pest moths. During the early years of pheromone identifications, many researchers thought that pheromones were single compounds, giving rise to a "magic bullet" theory which was generally accepted by most of the scientific community. The basis of this theory was that every insect produced a single compound that was chemically distinct from closely related insects which maintained isolation between species (Tumlinson and Teal, 1987). The identification of (Z)-7-dodecenyl acetate from the cabbage looper moth, *T. ni* (Berger, 1966) contributed to the acceptance of this assumption. The identification of single

components of the pheromones resulted from the use of non-discriminating bioassays used to identify pheromones which examined only part of the male behavioral repertoire. Major pheromone components often elicit responses in males, such as upwind flight. Additionally, chemical analytical methods of the time were also unable to resolve minute quantities of the minor components (Tumlinson and Teal, 1987). Nearly 20 years after the initial identification of (Z)-7-dodecenyl acetate, the major component of the *T. ni* pheromone, the reproductive behavior of *T. ni* males was more carefully scrutinized. From these observations, additional pheromone compounds were identified from the female cabbage looper (Bjostad et al., 1984; Linn et al., 1984). The pheromone blend of females of *T. ni* is now known to contain (Z)-7-dodecenyl acetate, dodecyl acetate, (Z)-5-dodecenyl acetate, 11-dodecenyl acetate, (Z)-7-tetradecenyl acetate and (Z)-9-tetradecenyl acetate. It is now known that the majority of moth pheromones which have been identified are composed of blends of compounds.

Hunt and Haynes (1990) reported that *T. ni* females release pheromone for an extended period of time, in bouts, during the scotophase beginning with a rapid release at the onset of scotophase and decreasing during the night. However, in field cage studies, males visited virgin females 3-8 hours after dark, during a 10-hour night (Lenczewski and Landolt, 1991). Interestingly, females were attracted to males at dusk (Landolt and Heath, 1990) and males released pheromone from their hairpencils in response to female pheromone or host plant odor. This demonstrates a dual strategy of mate-finding during different times of the night and a turn-about in the previously held perception that females passively released pheromone awaiting the arrival of the actively searching males. This kind of dual mating strategy may not be unique to the cabbage looper and may be widespread in other moths. Further investigation is needed in other species to determine the roles that each sex plays in the sexual strategies of lepidopterans.

Sex Pheromone of *Helicoverpa zea*

Roelofs et al. (1974) isolated (Z)-11-hexadecenal from *H. zea* abdominal tip extracts and demonstrated with electroantennograms (EAG) that this compound was stimulatory to male antennae. EAG is a sensitive method where compounds are delivered to antennal preparations to assay the olfactory response of sensory neurons to behaviorally active materials (Schneider, 1962). Traps baited with (Z)-11-hexadecenal caught significant numbers of males, establishing this compound as a sex pheromone (Roelofs et al, 1974). Ovipositor washes of *H. zea* females contained a blend of 16-carbon compounds including (Z)-11-hexadecenal, (Z)-9-hexadecenal, (Z)-7-hexadecenal and hexadecenal. Klun et al. (1980) demonstrated male precopulatory behavioral responses in cage bioassays with only the (Z)-11-hexadecenal and the (Z)-9-hexadecenal. Field trap studies showed that males were equally attracted to either of these 2 compounds or to all four components found in the female ovipositor extract.

Volatile collections obtained from *H. zea* pheromone glands contained all four compounds reported from abdominal tip extracts (Pope et al., 1984). The (Z)-11-hexadecenal was found in all females tested and made up over 90% of the blend. Hexadecenal was also commonly found, though much smaller quantities were present (5-7%). The other 2 components, (Z)-7-hexadecenal and (Z)- 9-hexadecenal were less commonly found at measurable levels. Volatile collections showed that peak production of pheromone occurred 1 to 2 hours after the onset of scotophase.

Sex Pheromone of *Heliothis virescens*

Roelofs et al. (1974) also reported that (Z)-11-hexadecenal was a pheromone for *H. virescens*. In addition, a 14-carbon compound, (Z)-9-tetradecenal was found in abdominal

tip extracts. EAG and field tests were used to measure the attractance of males to these compounds, though field tests were inconclusive. Another study confirmed that at a 16:1 ratio of (Z)-11-hexadecenal and (Z)-9-tetradecenal was present in *H. virescens* (Tumlinson et al., 1975). Later, Klun et al. (1980a) reported additional compounds in pheromone gland extracts. (Z)-11-hexadecenal was the major component comprising more than 80% of the blend. The other compounds identified by gas chromatographic analysis were (in order of abundance): hexadecenal, (Z)-11-hexadecen-1-ol, (Z)-9-tetradecenal, tetradecenal, (Z)-9-hexadecenal and (Z)-7-hexadecenal. Three additional alcohols, tetradecanol, (Z)-9-tetradecanol and hexadecanol, were also identified from gland extracts (Teal et. al., 1986).

Analysis of volatile collections from calling females of *H. virescens* revealed that six aldehydes were released from calling females. The aldehydes identified from volatile collection were (Z)-11-hexadecenal (60%), (Z)-9-tetradecenal (18%), tetradecenal (13%), hexadecenal (7%), (Z)-9-hexadecenal (1%) and (Z)-7-hexadecenal (0.6%) (Teal et. al., 1986). Behavioral analyses showed that all six of these components are important for close-range male reproductive behavior and a synthetic blend of all six compounds elicited a male response identical to the male response to calling females (Teal et al., 1986).

Endogenous Regulation of Pheromone Production

Endogenous Initiation of Pheromone Production

Riddiford and Williams (1971) were the first to suggest that neurohormonal mechanisms may be involved in the regulation of pheromone production in Lepidoptera. They reported that removal of the neurohemal organ complex of the corpora cardiaca-

copora allata (CC-CA) from females of *Hyalophora cecropia* and *Antheraea polyphemus* greatly reduced calling behavior. Implantation of the CC-CA after removal restored calling. They also found that the brain had to have intact neural connections with the CC-CA for calling to occur. This led them to hypothesize that the brain was releasing a hormone that stimulated the CC-CA to release a second hormone that affected calling and they assumed, pheromone production. In later studies, CC-CA homogenates, as well as hemolymph taken from calling *H. cecropia* females and injected into isolated abdomens, brought about normal calling behavior in 25-60% tested (Sasaki et al., 1983). In this same study, the role of the CC-CA in pheromone production was reevaluated. They found that the removal of the CC or CA did not affect calling behavior. However, they did find that the nervous system, from the brain to the terminal abdominal ganglion, had to be intact to maintain calling behavior. This suggests that the control mechanisms for calling in these moths are neural with some neurohormonal regulation, since the injection of CC-CA homogenates did stimulate calling.

Hollander and Yin (1982) postulated that pheromone production in the gypsy moth was neurally regulated. They found that pheromone production was interrupted by removal of the brain. Severing the nerve cord between the brain and the terminal abdominal ganglion (TAG) while leaving the brain intact also interrupted pheromone production. In a later study (Hollander and Yin, 1985), it was found that the brain, CC, CA or ovaries did not significantly affect either pheromone production or calling. Cutting the subesophageal connectives did not affect calling, but did interrupt pheromone release. Another study supported these findings. Removal of the CA did not affect the amount of disparlure (the gypsy moth pheromone), and cutting the nerve cord anterior to the TAG decreased the amount of pheromone produced and released (Tang et al., 1987). From these investigations, it appeared that neural factors are involved in pheromone release in the gypsy moth. It should be noted, however, that pheromone production and calling behavior

are likely independently controlled by two different mechanisms in most moths (Raina, 1993).

Raina and Klun (1984) provided the first definitive evidence for neurohormonal regulation of pheromone production in Lepidoptera. They showed that ligation between the neck and thorax of *H. zea* performed during the photophase, prevented the production of pheromone during the normal period of pheromone release of the subsequent scotophase. Extracts of the brain that were injected into the abdomens of ligated females during the period of pheromone production, stimulated production of normal amounts of pheromone in about four hours. In a later study (Raina et al., 1987), this pheromone-stimulating factor was isolated in the greatest amounts from subesophageal ganglia. The injection of homogenates of the brain and subesophogeal ganglion (the cephalic ganglia) into ligated females caused a rapid pheromonotropic response. Pheromone stimulating substances were also found in the brains of male *H. zea* and were present during both the scotophase and the photophase.

The pheromonotropic substance from *H. zea* brain-SEG was later determined to be a 33 amino acid peptide and was named pheromone biosynthesis activating neuropeptide (PBAN) (Raina et al., 1989). Soon after the identification of this PBAN (Hez-PBAN) (see Raina & Gäde, 1988 for peptide nomenclature), a similar neuropeptide was identified from *B. mori*, Bom-PBAN-I (Kitamura et al., 1989). A second pheromonotropic neuropeptide, Bom-PBAN-II, was also isolated from *B. mori*. This peptide differs from Bom-PBAN-I in having an additional amine terminal arginine residue, (Kitamura et al., 1990). Recently, a neuropeptide has been isolated and identified from the gypsy moth, Lyd-PBAN (Massler et al., 1994). These four PBANs are structurally similar with about 80% homology. All share the same 5 amino acid C-terminal sequence and are amidated. This C-terminal sequence is similar to a group of myotropic peptides, the pyrokinins,

which were isolated from cockroaches and locusts (Holman et al., 1986; Schoofs et al, 1991).

Members of the pyrokinin family of neuropeptides differ in amino acid length, but share the C-terminal sequence of FXPRLamide where X can be V, T. S or G. PBANs have S in the variable position of the terminal sequence or core. This sequence has been referred to as the active core, because when fragments of PBAN or other pyrokinins have been assayed for either pheromonotropic or myotropic activity, respectively, the minimal sequence necessary to elicit a physiological response is the C-terminal pentamer (Raina and Kempe, 1990; Nachman et al., 1986). The PBANs and two other types of peptides isolated from moths contain this pentamer and so all are classified as belonging to the pyrokinin group. The other lepidopteran peptides are the egg diapause hormones of *B. mori*, the Bom-DHs (Imai et al., 1991) and Pss-PT, a peptide with pheromonotropic activity isolated from the larvae of the army worm (Matsumoto et al., 1992).

With the similarities of structure among different pyrokinins, it is not surprising that cross-activity occurs. Bom-PBAN-I has myotropic properties in hind-gut preparations (Nachman and Holman, 1991) and some myotropins have pheromonotropic activities (Fonagy et al., 1992b; Kuniyoshi et al., 1992). These peptides were isolated using assays for specific physiological functions, but given the occurrence of cross-activities, pinpointing the specific function and mode of action for each peptide is a complex problem. Studies are now underway to determine the genes that encode for these peptides (Kawano et al., 1992; Ma et al., 1994) and as further information becomes available, the naturally occurring functions of individual peptides may become more clear.

Distribution and Mode of action of PBAN. The tissue with the greatest activity from which Hez-PBAN was isolated was the SEG of *H. zea* (Raina et al., 1987). Immunocytological studies with PBAN antisera have localized clusters of PBAN-immunoreactive cells in the SEG (Blackburn et al., 1992; Kingan et al., 1992). Axons

from these SEG cell clusters extend to the CC, the maxillary nerve and into the ventral nerve cord (Kingan et al., 1992). These findings would lead to the hypothesis that PBAN is released from the CC into the hemolymph. However, the hemolymph has very low pheromonotropic activity (Raina and Klun, 1984) and PBAN has never been isolated from hemolymph. However, injection of PBAN into the hemolymph does stimulate pheromone production. The reason why PBAN has not been found in hemolymph may be due to very small concentrations being present and also because the volume of hemolymph that can be removed from adult moths is only a small fraction of the circulating hemolymph (Raina, 1993). In most moths, PBAN is thought to act directly on the pheromone gland (Fonagy et al., 1992a; Jurenka et al., 1991b; Rafaeli and Soroker, 1989) by activating a plasma-membrane Ca^{2+} channel which regulates adenylate cyclase, and results in increased cellular cAMP levels.

There is also evidence that PBAN may be transported through the ventral nerve cord. Axons from the SEG project into the ventral cord and paired immunoreactive cells are found in all thoracic and abdominal ganglia except the terminal abdominal ganglion (TAG). Axons extending from the SEG into the VNC transverse the entire length of nerve cord and terminate in the TAG in both *H. zea* and *Manduca sexta* (Kingan et al., 1992; Davis et al., 1995). Studies on the gypsy moth strongly suggest neural involvement in pheromone production (Hollander and Yin, 1982, 1985; Tang, 1987) and it has been found that the VNC is the major route for PBAN transport in this insect (Masler et al., 1994; Thyagaraja and Raina, 1994). Studies conducted on *H. zea* indicated that transection of the ventral nerve cord disrupted normal pheromone production during the subsequent photophase (Teal et al., 1989). Injection of pheromonotropic substances into these females with cut nerve cords, brought about the production of pheromone. PBAN immunoreactivity, as well as pheromonotropic activity, has been found in the TAG of *H. armigera* (Rafaeli et al., 1991). PBAN is transported from the ventral nerve cord to the

TAG of the gypsy moth (Masler et al. 1993). Ample evidence exists to support the view that insects use both hemolymph and neural transport of PBAN.

Two moth species seem to be exceptions to the usual mode of action of PBAN seen in most other moths. The first is the redbanded leaf roller moth, where PBAN produced in the brain does not act directly on the pheromone gland, but instead stimulates the bursa copulatrix to release a different peptide that affects pheromone production (Jurenka et al., 1991a, Fabrias et al., 1992). The second example is *T. ni*, where PBAN appears to have no effect on pheromone production, though PBAN does appear to be present in the brain (Tang et al., 1989, 1991). Other studies on *T. ni* have shown that PBAN may play some role in pheromone release, though not in biosynthesis (Zhao and Haynes, 1993).

Endogenous Termination of Pheromone Production

Insects must terminate pheromone biosynthesis either at the end of the diel period of calling or during other times when mating in not appropriate. The production of pheromone is considered a "virgin" activity. After a mating takes place in many insects, pheromone production is no longer necessary and in short-lived insects, it is crucial to begin oviposition as soon as possible if eggs are to be deposited before death.

Male dipterans have been shown to produce substances which cause females to display mated types of behavior. For example, females of *Musca domestica* tend to mate only once (Riemann et al., 1967). The major factor causing this monogamy was shown to be a substance in the male seminal fluid which was passed to females during mating. This substance was present in the accessory glands of males and was depleted with excessive and repeated matings (Leopold et al., 1971). The substance also had an effect on the female brain, since decapitated or brain destroyed females would mate repeatedly. In other studies, autoradiography and electron microscopy revealed that the male accessory gland

secretion was transferred to the vaginal pouches of the female. It then was carried into the hemolymph and accumulated in the head region (Leopold et al., 1970). In the mosquito, *Aedes aegypti*, females mate repeatedly, but only become inseminated once (Craig, 1967). As in the housefly, the male mosquito produces a substance in the accessory glands, called matrone, that is passed to the female during mating. After mating, matrone has a permanent sterilizing effect on the female. It also increases oviposition behavior in the female. Craig tested 12 other species in the genera *Aedes*, *Anopheles*, and *Culex* and found that all had similar male produced substances. Matrone and JH, which has been shown to initiate receptivity, both act directly on the TAG which most likely secretes a second humoral factor (Gwadz, 1972).

Lepidopteran also produce male-borne factors which induce mated behavior in females. Truman and Riddiford (1971) demonstrated that successful matings in *H. cecropia* stimulated oviposition. Females mated to castrated males, but receiving a spermatophore, continued behaving as virgins, showing that some factor associated with the sperm caused the switch from virgin to mated behavior. Riddiford and Ashenhurst (1973) continued this study and established that when the male produced substance contacted the bursa of the female, it stimulated the secretory cells of the bursa to produce some blood-borne factor which may cause the release of a hormone to stimulate oviposition. Bursae or hemolymph from mated and virgin females were implanted into virgin females. Only the implants from mated females stimulated oviposition in virgin females. Virgins of *Manduca sexta* showed a short-term switch in behavior when receiving a sterile spermatophore. Since the response did not last, it was thought to be mechanically stimulated from the stretching of the bursa. To bring about a more long lasting effect, sperm or testicular fluids had to be present (Sasaki and Riddiford, 1984).

Termination of Pheromone Production in *Helicoverpa zea*

Mating in *H. zea* terminates the production of pheromone. When pheromone titers were measured for mated females, it was shown that the major pheromone component decreased by 93% in less than 2 hours after mating (Raina et al., 1986). When virgins were injected with the hemolymph of recently mated females, pheromone titer dropped substantially (Raina, 1988). If males were castrated in the larval stage, but the accessory glands were left intact, spermatophores of adult males still induced mated behavior in females. This indicates that the "mated-like behavior" is not sperm related, but is related to some compound in the accessory glands, since extracts of accessory glands injected into females causes a depletion in pheromone titer (Raina et al., 1989). They indicated that a hemolymph-borne factor probably terminated pheromone production as well as metabolizing any remaining pheromone. This substance was found to be a peptide and termed PSP (pheromonostatic peptide) (Kingan et al., 1995).

Pheromone titers of *H. zea* virgins decreased with age and decreased significantly by the onset of scotophase by the 4th day after emergence (Raina et al., 1986). It was initially hypothesized that this decrease in pheromone titer was due to decreasing levels of PBAN in the brain (Teal et al., 1990). This was tested by taking cephalic ganglia or brain extracts from 5-day-old (senescing) virgins and injecting the extracts into 3-day-old virgins during the photophase. These 3-day-old females produced the same amount of pheromone as those injected with brain extracts from donor insects of the same age (3-day-old virgins) (Teal et al., 1990). If brain extracts from younger females were injected into older females, a low titer of pheromone was produced. This established that PBAN levels in the brains of aging female virgins were adequate to induce pheromone production. Based on the results from the work of Riddiford and Ashenhurst (1973) that demonstrated the role of the bursa in inhibition of virgin behavior in *H. cecropia*, Teal et al. (1990) explored the

possibility that the bursa might be responsible for the decrease in pheromone titers in virgin *H. zea*. Bursa homogenates were prepared from older virgin females and were injected along with PBAN into younger females. After one hour, the pheromone titer was significantly lower than that of virgins injected with only PBAN. Bursa extracts from younger females had no significant effect on pheromone titer, while that of older females decreased pheromone titer by 50%. Therefore, the level of the bursa inhibitory substance increased with age.

Different tissues were tested for inhibitory activity. Activity was confined to the bursa, ovaries and hemolymph of senescing virgins. Increasing doses of the bursa extract showed a linear response and a dose of 2 FE of bursa extract completely suppressed the production of pheromone. Injection of the bursa extract 30 minutes before the injection of PBAN or at the same time with PBAN, completely suppresses pheromone production. Bursa extracts injected 30 minutes after PBAN decreased pheromone production by about 50%. Therefore, the bursa of aging virgin females contains substances that produce an effect that is similar to the male-borne factors transferred to females during mating.

Overview of the Regulation of Pheromone Production in Virgin Moths

There are multiple factors that influence the production and release of pheromone in moths. These factors include physiological as well as environmental effects. The age, mating status, period of day length, temperature, light intensity, air velocity and the surrounding vegetation all effect the amount and the time that pheromone is released (Raina, 1993). The work reported here addresses several of the factors that influence the production of sex pheromones in virgin females of Noctuidae, a family of moths with many members causing devastation and billions of dollars of crop losses each year. Three particularly destructive noctuid species are the corn earworm, *H. zea*, the tobacco

budworm, *H. virescens* and the cabbage looper, *T. ni*. The pheromone communication systems of all three of these species have been well studied and each species serves as an excellent model for insect pheromone studies.

The goals of this study are to understand several endogenous mechanisms that regulate the production of sex pheromone in virgin moths. These include: 1) the determination of structure and activity of the neuropeptides which stimulate pheromone production; 2) the development of methods to deliver these pheromonotropic peptides; 3) the mechanism of pheromone suppression in aging virgins and 4) the inter-relationship of pheromone suppression and oviposition stimulation.

Environmental factors also play an important role in pheromone regulation. Chemical signals from host plants may override or interact with the photoperiodic regulation of pheromone production by affecting the release of PBAN (Roelofs, 1995). Therefore, additional goals of this research were to determine the effect of host plants on pheromone production and release in the cabbage looper and to determine the role of crowding within a species as well as the effect of the presence of females from different species on reproductive events.

Each of the factors regulate different aspects of pheromone production and needs to be understood. After the specific mechanisms are determined, then the way that each interacts to affect the other will need to be examined, so that the entire story of how pheromone communication is regulated in moths can be determined. As new information is gathered, it may give researchers more insight as to how to break down the sexual communication of pest insects and utilize the disruption of sexual communication to keep pest populations under control.

CHAPTER 2
NEUROHORMONAL STIMULATION OF PHEROMONE PRODUCTION

Introduction

Numerous studies have been conducted on the isolation, identification and function of insect neuropeptides during the past decade (Holman et al., 1990). These studies have shown that neuropeptides regulate nearly all aspects of insect homeostasis. Neuropeptides have been isolated and identified from several orders of insects, yet a commonality of structure and function has been found among many of these peptides. For example, the pyrokinin family of neuropeptides is widespread in several insect orders and its members serve different physiological functions. Peptides belonging to this class were originally identified as myotropins from a cockroach and the locust (Holman et al., 1986; Schoofs et al., 1991). Although the members of this class of peptides have different numbers of amino acids (see Nachman et al., 1993), they all share the common C-terminal sequence, FXPRLamide (X=V,T,S or G). This pentamer is the active core required for myotropic activity in the cockroach hindgut assay (Nachman et al., 1986), and recent studies have indicated that bioactivity is related to a β–turn associated with this region of the molecules (Nachman and Holman, 1991; Nachman et al., 1993; Nachman et al., 1991).

Recently, another series of peptides sharing the pyrokinin C-terminal sequence have been isolated and identified from Lepidoptera (Imai et al., 1991; Kitamura et al, 1989; Kitamura et al., 1990; Matsumoto et al., 1990; Matsumoto et al., 1992; Raina et al, 1989). These peptides serve several functions in Lepidoptera including the induction of sex

pheromone production in adults (Raina et al., 1989; Kitamura et al., 1989; Kitamura et al., 1990), reddish colorization and melanization in larvae (Matsumoto et al., 1990) and the induction of diapause in silkworm moth eggs (Imai et al., 1991).

Interestingly, one of the pheromone biosynthesis activating neuropeptides (PBAN) identified from the silkworm moth, Bom-PBAN-I (Kitamura et al., 1989; see Raina and Gäde, 1988 for peptide nomenclature), has myotropic activity in the cockroach hindgut (Nachman and Holman, 1991). Also, some of the pyrokinins from the cockroach and locust originally isolated on the basis of their differing myotropic activities (Schoofs et al., 1994) stimulated sex pheromone production in females of the silkworm moth (Fonagy et al., 1992b; Kuniyoshi et al., 1992). The cross-activity of these peptides is, to some extent, expected because the C-terminal pentapeptide fragment common to both PBAN and the myotropins is required for activity in both the cockroach hindgut myotropic assay and the pheromonotropic assay (Nachman et al., 1986; Raina and Kempe, 1990).

The fact that members of the pyrokinin family have different numbers of amino acids and different sequences provides an opportunity to study the structural requirements of these molecules for biological activity. Although two other studies have shown that some of the naturally occurring pyrokinins will induce pheromone production in the silkworm moth (Fonagy et al., 1992b; Kuniyoshi et al., 1992), there are no comprehensive structure-activity relationship studies that assess the relative importance of the C-terminal active core on the induction of pheromone production. Here are the results of a comprehensive study in which the pheromonotropic activities were assessed for a series of naturally occurring pryokinins, two synthetic analogs with specific N-terminal changes, as well as the C-terminal fragments of some of these molecules in the corn earworm moth, *Helicoverpa zea*. The results show the importance of the X-position in the C-terminal portion, as well as the influence of the N-terminal portion of these molecules for biological activity.

Materials and Methods

Pupae of *H. zea* were obtained from a laboratory colony maintained at the Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA-ARS, in Gainesville, Florida. The pupae were separated by sex, and after eclosion the females were held in 4-L cages and provided with cotton wicks soaked with 5% sucrose solution. The insects were held at 25 ± 2 °C, 65 ± 3% relative humidity with a 12:12 (L:D) photoperiod. Virgin females were used for bioassays 1 and 2 days after eclosion during the 7th-8th hour of the photophase when endogenous levels of pheromone are very low (Teal et al., 1989).

Peptides were synthesized and purified as described elsewhere (Nachman et al., 1993). The sequences of the peptides used in assays are summarized in Table 2.1. Pheromone biosynthesis neuropeptide from *H. zea* (Hez-PBAN) was dissolved in physiological saline (Christensen et al., 1991) and diluted to concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 pmol/μl. Insects were injected through the side of the 3rd abdominal segment using a 26 ga. needle with 20μl of the above solutions. Each female received a dose of 0.06, 0.2, 0.6, 2, 6, 20, 60 or 200 pmol. After 1 hour of incubation, the terminal abdominal segments, which contain the pheromone gland, were excised and placed in 10μl of hexane containing 10ng each of internal standards (octadecane and nonadecane) used for quantitation and calculation of relative retention indices. The pheromone gland extracts were analyzed for the amount of (Z)-11-hexadecenal (Z11-16:AL), the major sex pheromone component of *H. zea* (Klun et al., 1980) by capillary gas chromatography. Hewlett-Packard 5890® gas chromatographs were equipped with splitless injectors, flame ionization detectors and 30m X 0.25mm (id) fused silica SPB 10® (polar) or SPB1® (apolar) columns (Supelco). Conditions of

chromatography were as follows: initial temperature = 60°C, injector operated in the splitless mode for 0.5 min, oven temperature increased by 30°C/min after 1 minute, final temperature = 190°C (SPB10) or 200°C (SPB1), helium carrier gas at a linear flow velocity of 18cm/s. A Nelson Analytical 3000® acquisition system (Cupertino, CA) was used to collect and analyze the data (Teal et al. 1989).

Table 2.1. Sequences of pyrokinins used in pheromonotropic bioassays. Underlined areas represent the shared amino acid sequence

Bom-DH C-terminal pentapeptide	FGPRL-amide
LPK C-terminal pentapeptide	FTPRL-amide
Bom-PBAN C-terminal hexapeptide	YFSPRL-amide
Lom-MT-II	EDGFTPRL-amide
[pGlu[1]]Lom-MT-II	pEDGFTPRL-amide
LPK	pETSFTPRL-amide
[Glu[2],Glu[3]] Lom-MT-III	REEPFVPRL-amide
Lom-PK-II	pESVPTFTPRL-amide
Hez-PBAN C-terminal decapeptde	SRTKYFSPRL-amide
Lom-PK	pEDSGDGWPQQPFVPRL-amide
Bom-DH-I[19Trp]	TDMKDESDRGAHSERGALWFGPRL-amide
Hez-PBAN	LSDDMPATPADQEMYRQDPEQIDSRTKYFSPRL-amide

After the dose response of Hez-PBAN was completed, the 2 pmol dose was chosen to use for comparison with the pyrokinins assayed here. Insects were also injected with saline alone as a control. Each peptide was prepared by dilution with saline as described above. Based on the dose response for the activity of Hez-PBAN, 6 doses were chosen to assess the pheromonotropic activity of the other pyrokinins. Females were injected with 20μl containing 0.06, 0.2, 0.6, 2, 6, 20 or 60 pmol. Each bioassay consisted of 1 dose of

several different peptides along with saline and Hez-PBAN controls. The amount of pheromone present in extracts of the pheromone glands of females from each group was compared with that produced by females injected with 2 pmol of Hez-PBAN during that particular bioassay. After all the bioassays were completed, the amount of pheromone produced by each insect was calculated as a percentage of the amount of pheromone produced by females treated with Hez-PBAN. Data were analyzed using one-way ANOVA and *t* -test with Statview™ (Brainpower, 1985). Variability was reported as standard error of the mean.

Results

Females injected with doses of Hez-PBAN from 0.06 to 2 pmol showed a linear increase in pheromone production ($r = 0.96$), but with doses from 2 to 200 pmol, there was no difference in the amount of pheromone produced ($F(4, 33) = 0.517$, $p > 0.25$) (Fig. 2.1). The 2 pmol dose of Hez-PBAN was the minimum dose required to stimulate production of the maximum amount of pheromone, so this dose was used as a positive control in all bioassays with the other pyrokinins as a basis for comparison of the pheromonotropic activity of each peptide.

The C-terminal decapeptide of Hez-PBAN showed little activity except at the highest doses of 20 and 60 pmol (Fig. 2.2a). At 20 pmol, the amount of pheromone produced was approximately 50% that of Hez-PBAN and the highest dose of 60 pmol induced the production of the same amount of pheromone as was induced by 2 pmol of Hez-PBAN. The C-terminal hexapeptide of Hez-PBAN has the same sequence as the C-terminal hexapeptide of Bom-PBAN (Table 2.1). This peptide had no activity except at the 20 and 60 pmol doses, but at the highest dose, the amount of pheromone produced was only 31% of that produced when Hez-PBAN was injected (Fig. 2.2a).

The entire molecule of the *B. mori* diapause hormone (Bom-DH-[19Trp]) showed activity similar to that of the Hez-PBAN decapeptide (compare Fig. 2.2a to 2.2b). However, the C-terminal pentapeptide of Bom-DH-[19Trp] showed no activity at any dose tested (Fig. 2b). In addition to the other doses assayed, a 200 pmol dose was tested and showed no activity (Z11-16:AL of 3.01 ng compared to 60.75 ng for 2 pmol dose of Hez-PBAN; n = 4). This was the only pyrokinin or pyrokinin fragment we tested that did not have pheromonotropic activity at any dose tested. The C-terminal pentapeptide of the cockroach myotropin, leucopyrokinin (LPK), had little activity except at the highest dose

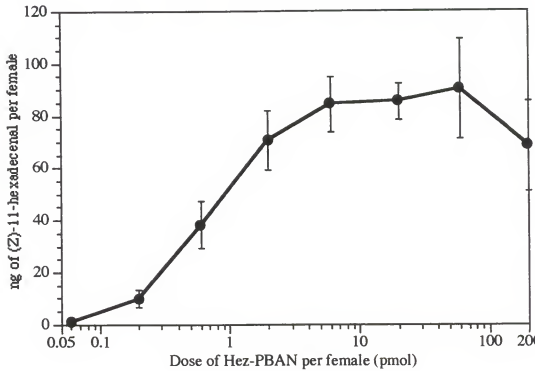

Figure 2.1. Effect of dose of Hez-PBAN on stimulation of *in vivo* pheromonotropic activity of in virgin females of *H. zea*. (Bars indicate SEM, n=8).

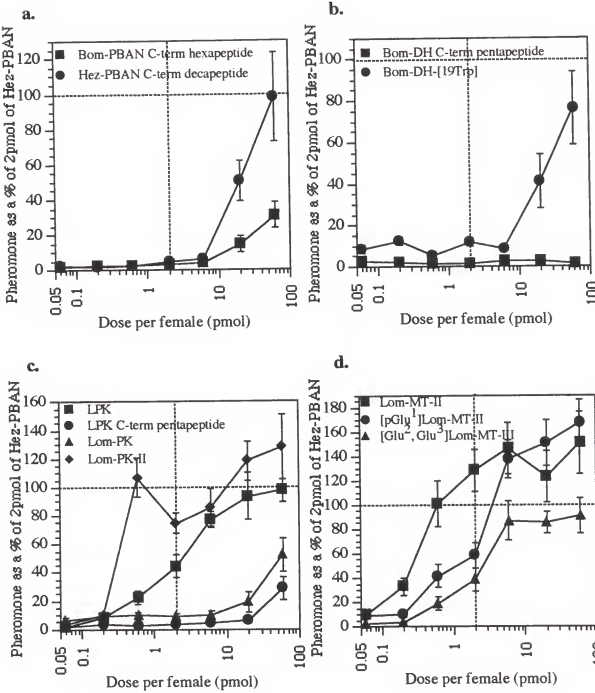

Figure. 2.2. Effect of dose of pyrokinin neuropeptides on simulation of *in vivo* pheromonotropic activity in virgin females of *H. zea*. The amount of pheromone induced by each peptide is expressed as a percentage of the amount of pheromone produced by females injected with 2 pmol of Hez-PBAN. Dashed lines represent the response (100%) from the 2 pmol dose of Hez-PBAN used for that particular bioassay. (Bars indicate SEM, n=8).

where 29% of the amount of pheromone was produced compared with Hez-PBAN (Fig. 2c). The entire LPK peptide, however, displayed an increase in activity with increasing dose and reached full activity by 20 pmol (t (14) = 0.366, 0.1p <.0.375). However, LPK was not as active as Hez-PBAN at lower doses (Fig. 2c).

Females injected with the locust myotropin or locustapyrokinin II (Lom-PK-II) produced as much pheromone when injected with 0.6 pmol as females treated with 2 pmol of Hez-PBAN (Fig. 2c). In contrast, Hez-PBAN treated insects produced approximately half as much pheromone when injected with the 0.6 pmol dose as they did when injected with 2 pmol (Fig. 2.1). Another locustapyrokinin (Lom-PK), induced production of pheromone but the amounts produced were lower than values obtained when 2 pmol of Hez-PBAN was injected (Fig. 2c).

All locustamyotropins (Lom-MTs) tested stimulated pheromone production at most doses greater than 0.06 pmol (Fig. 2d). [Glu^2,Glu^3]Lom-MT-III treated insects produced the same amounts of pheromone as 2 pmol of Hez-PBAN at doses of 6 pmol or greater (F (5,42) = 0.853, p > 0.25). The pheromonotropic response of females injected with [pGlu^1]Lom-MT-II was similar to [Glu^2,Glu^3]Lom-MT-III for doses up to 2 pmol, but at doses of 6 pmol and higher, the activity of [pGlu^1]Lom-MT-II exceeded that of Hez-PBAN (F (5, 42) = 2.226, .05 <p < 0.10). However, females receiving a 0.6 pmol dose of Lom-MT-II, produced the same amount of pheromone as insects treated with 2 pmol of Hez-PBAN and doses of 2 pmol and greater induced production of more pheromone than was produced by Hez-PBAN treated insects (Fig. 2d), although this was not statistically different from Hez-PBAN (F (7, 56) = 1.08, p > 0.25).

Discussion

The 5 amino acid C-terminal fragment of the PBAN molecule was shown to be the smallest fragment to induce a pheromonotropic response in *H. zea* (Raina and Kempe, 1990). The results indicate that the Bom-PBAN hexapeptide (identical sequence as Hez-PBAN hexapeptide) and Hez-PBAN decapeptide had little activity at doses in the physiological range of Hez-PBAN and that very high quantities were necessary to induce a pheromonotropic response. Raina and Kempe (1990) found a similar response with Hez-PBAN fragments and from these findings, it might be expected that small peptides are unable to elicit a full pheromonotropic response in *H. zea* at low concentrations. However, peptides that were 8-10 amino acids in length were as active or more active than the 33 amino acid Hez-PBAN molecule. In fact, the locust myotropins Lom-PK-II and Lom-MT-II produced a full pheromonotropic response at one-third the dose of Hez-PBAN and were as active at the 0.6 pmol dose as 2 pmol of Hez-PBAN. These neuropeptides, as well as Lom-MT-II with a *p* glu blocked N-terminus, were the most active neuropeptides that were tested. Each of these peptides had a threonine in the X-position within the active pyrokinin core (FXPRLamide). [*p* Glu^1]Lom-MT-II was less active than Lom-MT-II, so it does not appear as though the *p* glu enhances the activity of these peptides. Pyroglutamation may, however, provide resistance to enzymatic degradation in the hemolymph of the insect. A cockroach myotropin, LPK also has a threonine in the C-terminal variable position and at higher doses stimulated production of as much pheromone as Hez-PBAN; however, this peptide was not as active as Hez-PBAN at lower doses. The C-terminal pentapeptide of LPK was only 28% as active at the 60 pmol dose as Hez-PBAN. This is similar to the pheromonotropic effect of the Hez-PBAN C-terminal pentapeptide (Raina and Kempe, 1990). Therefore, the threonine in the X-position alone,

does not impart all of the superagonist activity of Lom-MT-II and Lom-PK-II.

[Glu^2,Glu^3]Lom-MT-III and Lom-PK both have valine in the variable position (X) of the C-terminal active core. Lom-PK, a 16 amino acid peptide, had little activity and was similar to the Hez-PBAN hexapeptide, whereas [Glu^2,Glu^3]Lom-MT-III, a 9 amino acid peptide, had full pheromonotropic activity at the 3 highest doses.

Interestingly, the C-terminal pentapeptide fragment of Bom-DH[19Trp], which has a glycine in the X-position of the active core was not active at any dose tested, including an additional dose of 200 pmol. The full 24 amino acid Bom-DH[19Trp] peptide, however, was about as active as the C-terminal Hez-PBAN decapeptide. The glycine in the C-terminal pentamer may not provide a rigid enough conformation to fit the receptor site for pheromone stimulation. The whole peptide, however, may force the C-terminus into a position where the required conformation is attained.

Although the entire Hez-PBAN molecule was much more active than either the PBAN hexapeptide or decapeptide C-terminal fragments, the results indicate that some of the smaller peptides are not only more active than the PBAN fragments tested, but in some cases are more active than the entire PBAN molecule. From conformational studies showing that a cyclic analog of the Bom-PBAN hexapeptide retained significant activity (Nachman et al., 1993), it is likely that the short active peptides are forming conformations that resemble that of Hez-PBAN at the receptor site. The cyclic analog, cyclo[NTSFTPRL], has a type I-ß turn in the active core region, and it is possible that the active peptides tested here are producing a similar structure at the receptor. Because C-terminal fragments of Hez-PBAN of 6 and 10 amino acids are not as active as Lom-PK-II, LPK, [pGlu]Lom-MT-II, Lom-MT-II or [Glu^2,Glu^3]Lom-MT-III, the amino acids in the rest of the Hez-PBAN molecule may be changing the conformation to one that more closely fits the receptor site. This is even more likely with Bom-DH-[19Trp], where the C-

terminal pentapeptide fragment had no activity, but the whole peptide changes the conformation so that a 60 pmol dose produces nearly full pheromonotropic activity.

Recently, cross-activity of members of the pyrokinin family has been demonstrated for pheromonotropic and myotropic activities. Kuniyoshi *et al.* (1992) showed that LPK, Lom-PK and Lom-MT-I induced production of pheromone in *B. mori* at lower doses than unoxidized Bom-PBAN-I or the C-terminal PBAN hexapeptide or pentapeptide fragments and that Lom-MT-II was as active as oxidized Bom-PBAN-I. The PBAN hexapeptide was as active as the most potent myotropin, LPK, in stimulating a myotropic response in isolated cockroach hindgut. Fonagy et al. (1992b) also demonstrated cross-activity in pheromonotropic assays for *B. mori* and *Spodoptera litura* with Lom-MT-II and Lom-MT-III as well as myotropic activity with Bom-PBAN-I on both *Locusta* oviduct and *Leucophaea* hindgut assays. Reported here is the first instance of cross-activity of pyrokinin neuropeptides for pheromone induction in *H. zea*. In addition, some of the insects treated with myotropic pyrokinins produced more pheromone than those treated with Hez-PBAN. Females injected with Lom-PK-II and Lom-MT-II at a dose of 0.6 pmol per female produced twice as much pheromone as females injected with the same dose of Hez-PBAN. The Lom-MT-II injected females also produced more pheromone at higher doses than that produced by Hez-PBAN treated insects.

Functional as well as structural cross-activity of neuropeptides shows that different events in the physiology of insects may be more complicated and inter-related than previously thought. Studies involving the structure-activity relationships of these peptides will aid in determining the mode of action and control of many physiological functions. The cross-activities and structural homology of neuropeptides among several insect orders shows that these peptides are highly conserved through evolution. Therefore, a comparison of the structural similarities of neuropeptides may also lead to a better understanding of the evolutionary processes involved in the physiology and development of insects.

CHAPTER 3
INDUCTION OF PHEROMONE PRODUCTION BY TOPICAL APPLICATION OF AN AMPHIPHILIC PSEUDOPEPTIDE MIMIC OF HEZ-PBAN

Introduction

Studies on the function and structural activity of pyrokinins, such as described in the previous chapter, will aid in the understanding of the physiological processes of pheromone production. However, several practical uses for pyrokinins have been and are being developed. Most moth species produce pheromone and are sexually active during the night-time hours, and though the time period that each individual female produces pheromone is predictable, the exact time cannot be pinpointed. Injection of PBAN during the photophase induces the production of pheromone within an hour (Martinez and Camps, 1988; Teal and Tumlinson 1989), so that insects can be induced to produce pheromone simultaneously, predictably, and at any time that is convenient to the researcher. This ability to artificially induce the production of pheromone at will makes PBAN a powerful tool for several types of studies.

For example, PBAN was used as a tool in the identification of the pheromone components of *Plodia interpunctella*. (Teal *et al.*, 1995). Injection of PBAN has also proved useful in studying the biosynthetic pathways for pheromones (Fang et al., 1992, 1995a). Continuous injection of PBAN has been shown to deplete the amount of pheromone produced by *Manduca sexta* by decreasing the triacylglycerides which contain the precursors of pheromone components (Fang *et al.*, 1995b). Continuous stimulation of pheromone production by PBAN has led to inaugural studies on the development of

methods for delivery of this peptide as potential control agents. The rationale behind these studies is that continuous stimulation would lead to low levels of pheromone being released and bring about a disruption in the ability of males to locate females.

Raina et al. (1994) conducted a study utilizing this idea by feeding PBAN to moths. They found that starved *H. zea* females fed PBAN and PBAN analogs in sucrose solution during the photophase, produced pheromone. Although the amounts of pheromone produced were not as great as that induced by injection of PBAN, radiolabelling studies showed that PBAN analogs did cross the gut wall into the hemolymph and affected pheromone production.

In the study presented here, the most active of the pyrokinins from the previous chapter, Lom-MT-II, was modified at the N-terminus with a hydrophobic moiety, 6-phenylhexanoic acid. The underlying concept is that the addition of a hydrophobic character to a portion of the peptide would impart an overall amphiphilic nature to the molecule and thereby facilitate penetration through the cuticle. In addition, the amphiphilic nature of this pseudopeptide should afford greater solubility in a wider range of solvents than the parent or naturally occurring peptide, and allow for the development of topical application delivery systems for insect neuropeptides. The objectives of this study were to determine if such an alteration to a peptide would allow the peptide to retain its activity, and if so, if the amphiphilic pseudopeptide would in fact cross the cuticle more readily than naturally occurring peptides.

Materials and Methods

Pupae of *Heliothis virescens* were obtained from a laboratory colony maintained at the Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA-ARS, in Gainesville, Florida. The pupae were separated by sex and the females held in 4-L cages

and provided with cotton wicks soaked with 5% sucrose. The *H. virescens* females were held under the same conditions as *H. zea* in Chapter 2. Virgin females were used for bioassays 2 and 3 days after eclosion during the 7th-8th hour of the photophase when endogenous levels of pheromone are low (Teal et al., 1993).

Pheromonotropic Activity of [6-Pha-ala]Lom-MT-II

Peptides were synthesized and purified as described elsewhere (Nachman et al., 1993) and 6-phenylhexanoic acid-locustamytotropin-II ([6-Pha-ala^0]Lom-MT-II) was provided by Dr. Ronald Nachman of the Veterinary Entomology Research Unit, FAPRL, USDA-ARS, College Station, Texas. The activity of [6-Pha-ala^0]Lom-MT-II was first determined by an injection bioassay similar to chapter 2. Hez-PBAN and Lom-MT-II (for structures, refer to Table 2.1) were compared to [6-Pha-ala]Lom-MT-II for pheromonotropic activity. A series of dose responses was conducted for each peptide. The peptides were dissolved in water and diluted to concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 pmol/μl. Insects were injected through the side of the 3rd abdominal segment using a 26 ga. needle with 20μl of the above solutions. Each female received a dose of 0.06, 0.2, 0.6, 2, 6, 20, 60 or 200 pmol.

After 1 hour of incubation, the terminal abdominal segments, which contain the pheromone gland, were excised and placed in 10μl of hexane containing 10ng each of internal standards (octadecane and nonadecane) used for quantitation and calculation of relative retention indices. The pheromone gland extracts were analyzed for the amount of (Z)-11-hexadecenal (Z11-16:AL), the major sex pheromone component of *H. virescens* (Klun et al., 1979) by capillary gas chromatography. Hewlett-Packard 5890® gas chromatographs used for analyses were equipped with splitless injectors, flame ionization detectors and 30m X 0.25mm (id) fused silica SPB 10® (polar) or SPB1® (apolar)

columns (Supelco). Conditions of chromatography were as follows: initial temperature = 60°C, injector operated in the splitless mode for 0.5 min, oven temperature increased by 30°C/min after 1 minute, final temperature = 190°C (SPB10) or 200°C (SPB1), helium carrier gas at a linear flow velocity of 18cm/s. A Nelson Analytical 3000® acquisition system (Cupertino, CA) was used to collect and analyze the data (Teal et al. 1989).

Topical Application of Peptides in a Carrier Solvent

Hez-PBAN and [6-Pha-ala^0]Lom-MT-II were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 10, 50, 100, 500 and 1000 pmol/μl. The cuticular scales along the lateral side of the abdomen were removed with a 1 ml plastic pipet tip attached to house vacuum with a rubber hose. A low vacuum was applied to the apparatus and the pipet tip was gently passed over the cuticle until the scales were drawn by suction. After the scales were removed, 1μl of peptide solution was applied to the surface of the cuticle and smoothed evenly over the prepared surface. The insects were allowed to incubate 1 hour and then the pheromone gland was excised and the amount of pheromone determined by the method described above.

Comparison of Solvents for Topical Application

[6-Pha-ala^0]Lom-MT-II and Hez-PBAN were prepared in different solvents at a concentration of 500 pmol/μl. The solvents used were 80% acetone:water, and 80% acetonitrile:water, DMSO, and water. The scales were removed from the lateral side of the abdomen and the peptides were then topically applied to the cuticle. Each solvent tested consisted of 3 treatments: a blank, Hez-PBAN and [6-Pha-ala^0]Lom-MT-II. One hour

after the application of the test solutions, the glands were excised and the amount of pheromone was determined.

Dose Response of [6-Pha-ala^0]Lom-MT-II Applied Topically in Water

The amphiphilic peptide, [6-Pha-ala^0]Lom-MT-II, was dissolved in water and solutions at concentrations of 5, 10, 25, 50, 75, 100, 250, 500, 750 and 1000 pmol/μl were prepared. The scales were removed from the insects and the solutions applied topically to the lateral side of the abdomen. The amount of the pheromone present in the gland was determined after a 1 hour incubation period.

Topical Application of Peptides for Different Periods of Incubation

Hez-PBAN and [6-Pha-ala^0]Lom-MT-II were prepared in water at a concentration of 100 pmol/μl. The insects were prepared as described earlier and either water alone, Hez-PBAN or [6-Pha-ala^0]Lom-MT-II was topically applied. For each treatment (water, Hez-PBAN or [6-Pha-ala^0]Lom-MT-II) the insects were allowed to incubate for either 15 minutes, 30 minutes, 1 hour, 2 hours or 3 hours. The amount of pheromone present in the gland was determined after each incubation time.

Data were analyzed using one-way ANOVA and *t* -test with InStat® (Graphpad,1993). Variability was reported as standard error of the mean.

Results

Pheromonotropic Activity of 6-Pha-ala[0]Lom-MT-II

When injected, Lom-MT-II had greater activity than Hez-PBAN at all but the highest doses (Figure 3.1), similar to the results seen with *H. zea* (see Figure 2.2d in Chapter 2). At the highest doses of 60 and 200 pmol/female, the amount of pheromone was the same for all 3 treatments (F (5, 33) = 1.101, p = 0.378). The addition of

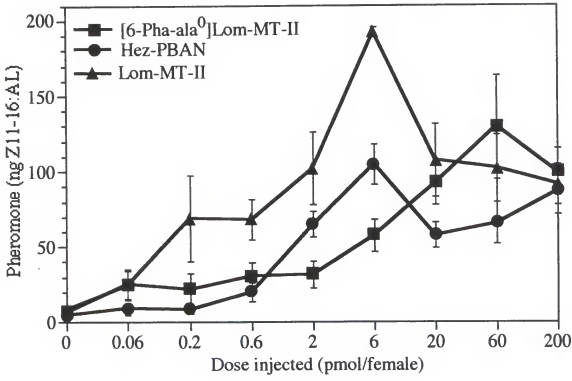

Figure 3.1. Dose response for injection of [6-Pha-ala[0]]Lom-MT-II, in comparison to Lom-MT-II and Hez-PBAN, on the stimulation of pheromone production by virgin females of *H. virescens*. (Bars indicate SEM, n=8).

6-phenylhexanoic acid to the parent Lom-MT-II peptide, did reduce pheromonotropic activity compared to Lom-MT-II for doses up to 6 pmol. The pheromonotropic response to 20 pmol of Lom-MT-II and [6-Pha-ala^0]Lom-MT-II was the same (t (10) = 0.557, p =0.590), both being greater than Hez-PBAN at the same dose.

Although [6-Pha-ala^0]Lom-MT-II was not as active as Lom-MT-II at lower doses, it was comparable to PBAN. As doses increased to 20pmol and above, [6-Pha-ala^0]Lom-MT-II treated insects produced as much pheromone as those injected with Hez-PBAN and Lom-MT-II.

Topical Application of Peptides in a Carrier Solvent

Insects treated topically with [6-Pha-ala^0]Lom-MT-II and Hez-PBAN in DMSO produced pheromone (Figure 3.2). However, insects treated with Hez-PBAN did not produce pheromone with a dose less than 50 pmol/female. In contrast, insects treated with [6-Pha-ala^0]Lom-MT-II, synthesized pheromone at 5 and 10 pmol doses. Insects injected with Hez-PBAN produce appreciable amounts of pheromone at a dose of 6 pmol (Figure 3.1). Therefore, although Hez-PBAN is able to pass through the cuticle in DMSO, higher doses are required for topical application than injection, to elicit a pheromonotropic response. On the other hand, lower doses of [6-Pha-ala^0]Lom-MT-II topically applied in DMSO produced results similar to injection of [6-Pha-ala^0]Lom-MT-II (Figure 3.1). At doses from 100 to 1000 pmol/female, injects treated with [6-Pha-ala^0]Lom-MT-II produced more pheromone than Hez-PBAN treated insects. However, differences were not statistically significant (F (5, 19) = 0.613, p = 0.692).

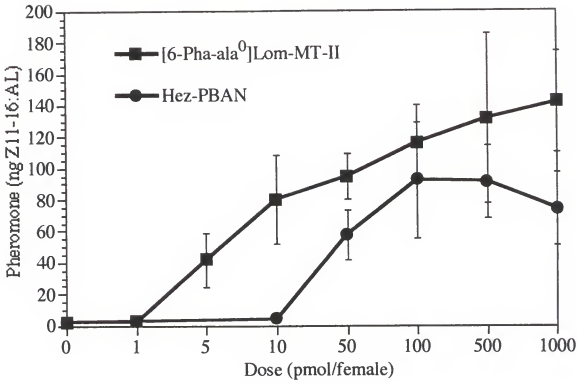

Figure 3.2. Effect of dose of 6-Pha-ala^0]Lom-MT-II and Hez-PBAN, on the stimulation of pheromone production by virgin females of *H. virescens*. Both peptides were dissolved in DMSO and topically applied to the lateral side of the abdomen after cuticular scales were removed (Bars indicate SEM, n=4).

Comparison of Solvents for Topical Application

One dose was chosen, 500 pmol/female, to compare different solvents for topical application of peptides. DMSO, 80% acetonitrile and 80% acetone flowed easily over the "de-scaled" cuticle. Water tended to ball up due to the wax layer of the cuticle, and was

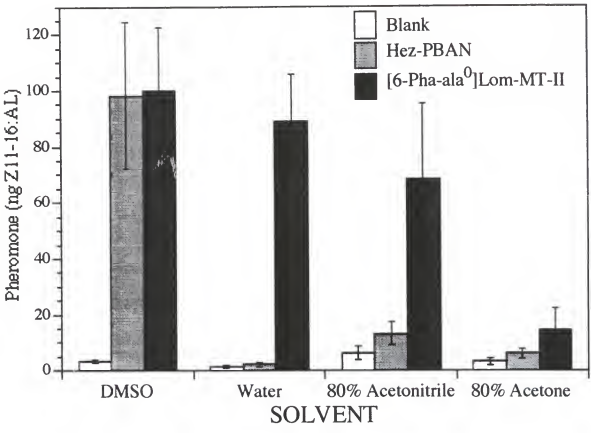

Figure 3.3. Pheromonotropic response of *H. virescens* females after topical application of a solvent blank, Hez-PBAN or [6-Pha-ala^0]Lom-MT-II in either DMSO, water, 80% acetonitrile or 80% acetone. (Bars indicate SEM, n=5).

difficult to apply as a blank or with Hez-PBAN dissolved in it. When [6-Pha-ala0]Lom-MT-II was dissolved in water at concentrations of 100pmol/μl and above, however, the amphiphilic nature of the molecule reduced the surface tension of the water so that it wetted the cuticle much like the other solvents. The activity of both [6-Pha-ala^0]Lom-MT-II and PBAN in 80% acetone were low (Figure 3.3) and not significantly different from each other (t (8) = 1.024, p =0.336). [6-Pha-ala^0]Lom-MT-II dissolved in 80% acetontirile was more active than PBAN in that solvent. When water was used as a solvent, a dramatic difference was seen between [6-Pha-ala^0]Lom-MT-II and PBAN (Fig. 3.3). The activity of [6-Pha-ala^0]Lom-MT-II dissolved in water or 80% acetonitrile was similar. Therefore, because water was a good solvent for topical application of [6-Pha-ala^0]Lom-MT-II, but a poor solvent for PBAN, water was chosen as the solvent to be used in further assays. Topical application of Hez-PBAN showed appreciable activity only in DMSO. This demonstrates that [6-Pha-ala^0]Lom-MT-II is crossing the cuticle independent of the solvent to stimulate pheromone production. DMSO is a good solvent for either peptide (Fig. 3.3).

Dose Response of [6-Pha-ala^0]Lom-MT-II in Topically Applied in Water

[6-Pha-ala^0]Lom-MT-II applied topically in water showed significantly more pheromonotropic activity than the blank by doses of 10pmol/μl (t (18) = 2.60 p =0.018) (Fig. 3.4) and above. Activity increased in a linear fashion to 100 pmol/μl (r = 0.98). Doses from 100 to 1000 pmol/μl had no difference in activity (F (4, 36) = 0.94, p = 0.453).

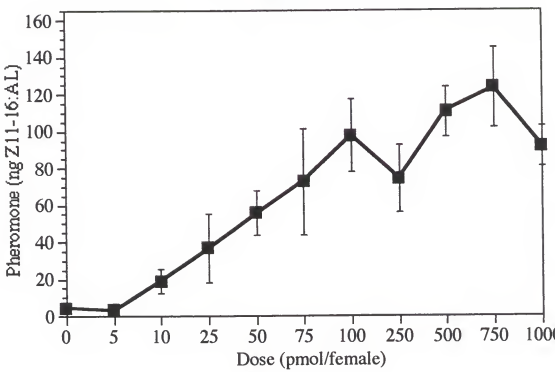

Figure 3.4. Dose response for topical application of [6-Pha-ala^0]Lom-MT-II in water for pheromonotropic activity in *H. virescens* females (Bars indicate SEM, n=10).

Topical Application of Peptides for Different Periods of Incubation

[6-Pha-ala^0]Lom-MT-II begins crossing the cuticle and stimulating the production of pheromone within 15 minutes after topical application in water (Fig. 3.5). Between 1 and 3 hours, the activity begins to gradually decline. Hez-PBAN showed some activity during the first 30 minutes after application, but very little after 1 hour.

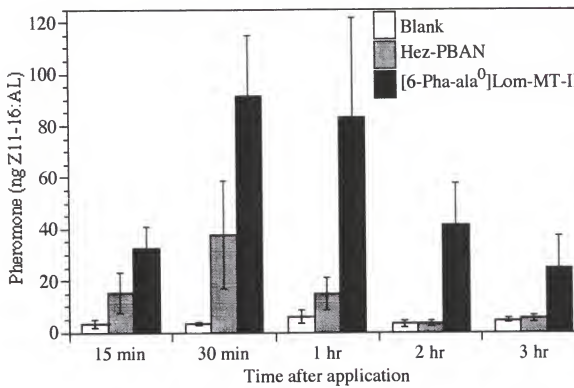

Figure 3.5. Topical application of 100 pmol doses of Hez-PBAN and [6-Pha-ala^0]Lom-MT-II in water. The amount of pheromone in the gland was evaluated after different time periods of incubation.

Discussion

The results clearly demonstrate that the N-terminus of peptides can be altered with nonpeptide moieties and retain pheromonotropic activity (Fig. 3.1). A recent study demonstrated that the structure of peptides can be mimicked by nonpeptide groups to produce conformationally similar analogs (Nachman et al., 1995). In this ground-breaking study, the three N-terminal amino acid residues were removed from the pyrokinin

pentamer active core, leaving only the RL-amide C-terminus and were replaced by a carbocyclic proline-mimetic moiety. The activity was retained in hind-gut myotropic assays with activity similar to that of the naturally occurring C-terminal pentamer (FXPRLamide). This pseudopeptide retained many of the structural characteristics of the pentamer core. This study represents the first development of pseudo or nonpeptide mimetic analog for insect neuropeptides (Nachman et al., 1995).

In the present study, the active core of the pentamer was retained, but a hydrophobic moiety was added to the N-terminus. This alteration of an extremely active small peptide, Lom-MT-II, allowed the novel pseudopeptide, [6-Pha-ala^0]Lom-MT-II to retain much of the native peptide's pheromonotropic activity. The properties of [6-Pha-ala^0]Lom-MT-II are quite different than that of the naturally occurring pheromonotropic peptide, PBAN. The amphiphilic nature of the peptide not only allows it to be soluble in water, but breaks the surface tension of the water so that it can be readily applied to the cuticle. Water alone or PBAN dissolved in water are both very difficult to apply to the cuticle because the great surface tension of water permits very little adherence to the waxy surface.

DMSO has been used as a carrier solvent for numerous types of studies in many organisms. DMSO is often used to apply pheromone precursors to both *in vitro* and *in vivo* insect preparations to study pheromone biosynthesis. PBAN dissolved in DMSO penetrates the cuticle and stimulates pheromone production. The technique of applying PBAN in DMSO provides a non-invasive approach for introducing PBAN into the insect and may provide useful for some studies because of the limited disruption to the insect. [6-Pha-ala^0]Lom-MT-II, however, is active at lower doses than PBAN in DMSO and has similar activity as injection assays making it even better and more economical to use than PBAN.

Not only is [6-Pha-ala^0]Lom-MT-II active in a wider variety of solvents than PBAN, but it acts longer and lasts for up to 3 hours after application. Although field applications are not feasible at this point, this study does demonstrate that PBAN agonists can be developed with different properties than native peptides. This is a pilot study, and only the beginning of a promising new method of peptide delivery. New PBAN agonists, like [6-Pha-ala^0]Lom-MT-II, may have many uses for physiological studies. Nonpeptides which retain physiological activity, and can be used in a variety of solvents with the ability to freely cross the insect cuticle, may allow for studies that are not possible with naturally occurring neuropeptides.

CHAPTER 4
HOST PLANT EFFECTS ON PHEROMONE PRODUCTION

Introduction

For some time, plant volatiles have been suspected to be involved in the induction of sex pheromone production, but only recently has there been definitive evidence for this. Several corn silk volatiles, including ethylene, stimulated pheromone production in *H. zea* (Raina et al., 1991). Interestingly, the first generation progeny of field collected larvae were used in this study, as colonized *H. zea* produce pheromone with or without the presence of host plants and show no increase in pheromone production when host plants are present.

Female cabbage loopers, *T. ni,* in colony, have responded to host plant volatiles. Both virgin females and males of *T. ni* were attracted more to the opposite sex when a bouquet of cotton foliage was present (Landolt et al., 1994). Males were more attracted when the cotton odor was passed over the females first than when female odor was passed through cotton volatiles, suggesting that the females may be affected by the cotton odors to release a pheromone more attractive to males.

This study was designed to discover whether the production of pheromone in females of *T. ni* is affected by the presence of a host plant under greenhouse conditions. The amount of pheromone present in the gland was evaluated, as well as the amount of pheromone released as volatile emissions. The response of males to these emissions was monitored to determine if males had a preference for females on cotton.

Materials and Methods

Pupae of *T. ni* were obtained from a laboratory colony maintained at the Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA-ARS, in Gainesville, Florida. The pupae were separated by sex and were held in screen cages (60 X 60 X 60 cm) until adults emerged and then the pupae were transferred each day to clean cages. The adults were provided with cotton wicks soaked with 5% sucrose solution and fresh water. The cages were held in vented areas in a greenhouse and each sex of moth was kept in a separate room. The insects were used for the experiments 3 and 4 days after eclosion. All experiments took place in a greenhouse under ambient environmental conditions.

Cotton plants were grown in 4-l pots in a commercial potting soil mix in a screened-in area outside. The plants were grown from seed (Germaine 510 variety) and were used for assays when the plants were 5-8 weeks old.

The amount of Pheromone Present in Glands Over a 24 hour Period

Five females were placed in a small plexiglass cage (14 X 14 X 28 cm) with 10 cm holes on the ends and tops covered with fine mesh nylon fabric to allow airflow. Twelve of these cages were supplied with 2 small plastic cups (3 ounce Dixie® cups cut to a height of about 3 cm) containing cotton balls soaked with either 5% sucrose or water. The 12 small cages were placed in a rectangular wind tunnel made of 0.64 thick acrylic plexiglass. The dimensions of the wind tunnel were 30.2 X 30.2 X 122 cm and airflow was provided by an air purification system at the upwind end of the tunnel, consisting of charcoal filters and diffusers. A reducing duct was attached to the downwind end of the tunnel and an

exhaust blower assembly set the average wind speed at 0.11 m/sec (for a detailed description of the system see Heath et al., 1993). The air purification system of the wind tunnel allowed only clean air to pass over the small cages within. Every 2 hours for a 24 hour period, a cage was removed from a random position inside the tunnel, and the terminal abdominal segments of each female in that cage were excised and extracted with 30 µl of hexane containing 30ng each of internal standards (octadecane and nonadecane).

The amount of the major pheromone component, (Z)-7-dodecenyl acetate (Z7-12:Ac) (Berger, 1966) was analyzed by capillary gas chromatography. Hewlett-Packard 5890® gas chromatographs equipped with splitless injectors, flame ionization detectors and 30m X 0.25mm (id) fused silica SPB 10® (polar) or SPB1® (apolar) columns (Supelco). Conditions of chromatography were as follows: initial temperature = 60°C, injector operated in the splitless mode for 0.5 min, oven temperature increased by 30°C/min after 1 minute, final temperature = 190°C (SPB10) or 200°C (SPB1), helium carrier gas at a linear flow velocity of 18cm/s. A Nelson Analytical 3000® acquisition system (Cupertino, CA) was used to collect and analyze the data (Teal et al. 1989).

Another set of small cages and a second wind tunnel were set up in exactly the same manner, except that each small cage was provided with a small glass vial (50 ml) containing a freshly cut stem of cotton. The 2 wind tunnels were placed side by side in the greenhouse, so that the moths would experience similar environmental parameters, but the charcoal imbedded filters at the upwind end of the wind tunnels kept the plant volatiles from affecting the females in cages without plants. The females held with cotton stems were sampled every 2 hours for a 24 hour period for the presence of Z7-12:Ac in the glands.

Volatile Emission of Pheromone From Females Held with Cotton Bouquets

An automated volatile collection system was placed in the greenhouse next to the wind tunnels used for pheromone gland analysis, so that the amount of pheromone being released from females could be monitored at the same time and under the same conditions. The volatile collection system used in this experiment consisted of 3 parts as described elsewhere (Heath and Manukian, 1994). The first is the volatile collection chamber (Fig. 4.1), which is a sealed modification of the guillotine system collection assembly (Heath and Manukian, 1994) and similar to a smaller push-pull system (Heath et al., 1993) in which insects are held and purified air is pushed at one end (bottom) and pulled through volatile collector traps, or glass tubing containing the adsorbent Super-Q® (Alltech) at the top (Heath and Manukian, 1994). The other parts of the system are an automated volatile collection system and an environmental data monitoring system which are described elsewhere (Heath and Manukian, 1994).

Two volatile collection chambers were placed next to each other and each was supplied with small plastic cups described earlier with sucrose solution and water. Five *T. ni* females were placed in one chamber, and another 5 were placed in a second chamber which contained a bouquet of 2 cotton stems in a 150 ml glass bottle. Another glass bottle was placed in the no-plant chamber. Each chamber was equipped with 8 Super-Q filters and volatile collections were made every 2 hours beginning at 6:00 PM and ending the following morning at 10:00 AM. During the first series of collections in 1994, half of the volatiles was collected onto filters and the other half was split off and run to a third wind tunnel. In these studies, the inlet air flow was 1 l/min and the suction was set at 500ml/min. In subsequent studies during 1995, the outlet to the wind tunnel was closed and all of the volatiles were collected and the suction was set at 1 l/min.

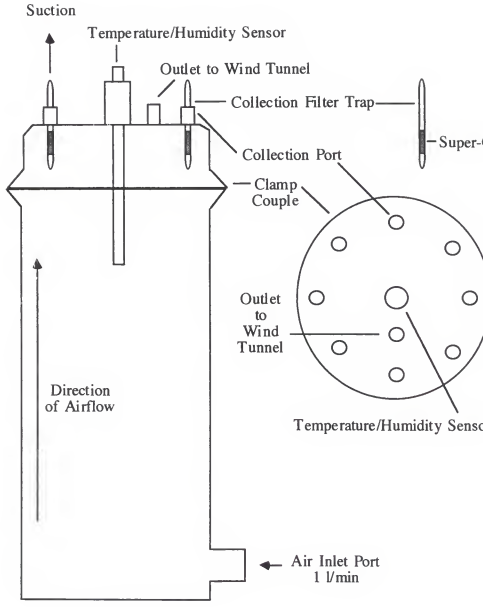

Figure 4.1. Diagram of the collection chamber of the automated volatile collection system.

After the collection period, the Super-Q filters were extracted with 100μl of methylene chloride and 50 ng of the internal standard, pentadecyl acetate, was added to each sample. The samples were analyzed for Z7-12:Ac as described earlier. The results were adjusted to reflect the amount of pheromone released for 1 female per hour.

Male Response to Volatile Emissions from Females

As described in the previous section, during 1994, half of the volatiles from each collection chamber were split off and run to a large wind tunnel (0.75 X 0.75 X 2.5 m) with air speed of .2m/sec. Teflon tubing (id .64 cm) was run from the collection chambers to dual outlets of copper tubing which were spaced 25 cm apart. Each outlet provided volatile emissions from one chamber, either females alone or females with a cotton stem bouquet. Five *T. ni* males were released at the downwind end of the wind tunnel every 2 hours during the period in which volatiles were collected from females. The males provided a real time biological response to correlate with the amount of pheromone collected and analyzed from females. The males were videotaped using a video system described earlier (Heath et al., 1993). The number of contacts made with each outlet were recorded when the videotape was reviewed.

Time Frame of Experiments

Studies determining the amount of pheromone in the glands, the amount of pheromone released by volatile collection analysis and the response of males in a wind tunnel were all conducted in tandem for August, September and October of 1994. The following year, only volatile collection analysis of pheromone release was evaluated beginning at the end of May and continuing until the middle of September.

Data were analyzed using *t* -test with InStat® (Graphpad,1993). Variability was reported as standard error of the mean.

Results

Tandem experiments for the amount of pheromone present in the gland, the amount of pheromone released and collected in the volatile collection system, and the response of males to these volatiles were conducted in late August and in mid September and mid October. The results from different times were highly variable, so each assay was evaluated alone and the difference between females alone or on cotton was the basis for comparison for each particular date.

T. ni females stored a large amount of pheromone in their glands (hundreds of ng), but the amounts released that were detected in volatile collections were small (ca 10 ng or less). There was a difference in both the amount and time of release for females with or without plants during August, 1994 (Fig. 4.2). When no plant was present, females released little pheromone at the beginning of the scotophase, began releasing pheromone at the middle of the scotophase around 2:00 AM, and the release peaked towards dawn. Just the opposite response was seen in females held on cotton bouquets, who released large amounts of pheromone right before and after dusk and most of the pheromone was released during the first half of the scotophase.

The amount of pheromone extracted from glands inversely mirrored that seen in volatile release (Fig. 4.2). There was a drop at the very beginning of the scotophase in the amount of pheromone in the glands of females held on plants with an accompanying release of pheromone during the same time period. The pheromone in the glands of females held without plants, did not begin to decline until the middle of scotophase, at the same time that volatile release began.

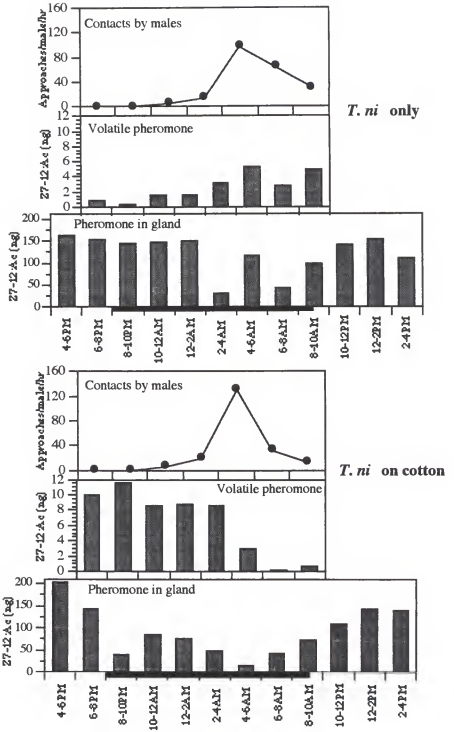

Figure 4.2. A comparison of the amount of pheromone present in glands, the amount released and the male response to volatiles emitted from *T. ni* females alone or on a bouquet of cotton. The study took place on August 28-29, 1994. The black bar represents the period of scotophase.

Interestingly, males were most responsive towards dawn and even when large amounts of pheromone were released from the females held on plants early in the night, the males did not show much attraction (Fig. 4.2). The males slightly favored the females without plants towards dawn, at which time the pheromone release was peaking.

During September, there was not such a clear-cut difference between females with and without plants as during August (Compare Fig. 4.2 and 4.3). Females on plants did not release pheromone as early as in August, but did release pheromone over the entire scotophase and showed a gradual increase until mid-scotophase and then a gradual decline towards dawn (Fig. 4.2). Those without plants, released pheromone earlier than in August at 10:00 PM -12:00 AM, but showed a large dip in the center of the scotophase and then again started producing towards dawn. It could be suspected that this release showing a bimodal peak curve, could be due to some artifact of collection or handling, but the male response corresponds with it.

Males showed a preference for females without plants, and there was a big decline in male activity during the valley between the peaks of the bimodal pheromone release curve, and even though females on plants were producing a relatively large amount of pheromone at the time, the males did not respond to either outlet (Fig. 4.3).

During October, females that were not on plants, produced more pheromone than females with cotton bouquets (Fig. 4.4). Moths without plants released pheromone during the second half of scotophase, while females on cotton released pheromone over most of the scotophase with peak release occurring during the middle. The amount of pheromone in glands did not correspond as well to volatile release of pheromone as the August and September trials. Females without plants had a decline in the pheromone in glands at the beginning of the scotophase with no corresponding release of pheromone. The amount in glands then increased toward the middle of the scotophase and declined again during the second half, matching the release of females in the volatile collection chamber.

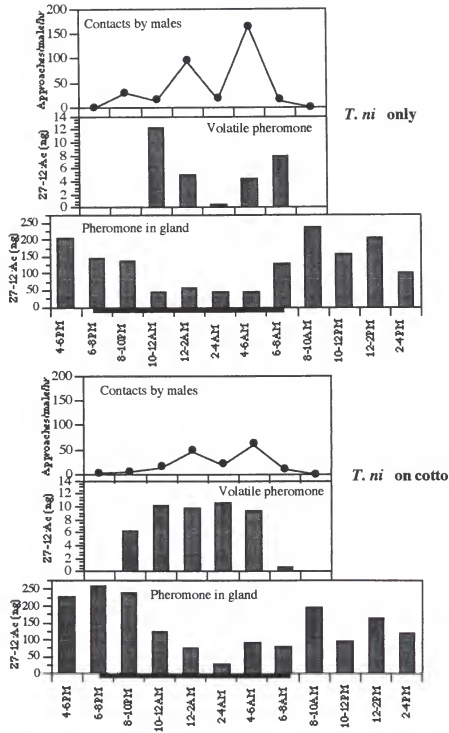

Figure 4.3. A comparison of the amount of pheromone present in glands, the amount released and the male response to volatiles emitted from *T. ni* females alone or on a bouquet of cotton. The study took place on September 18-19, 1994. The black bar represents the period of scotophase.

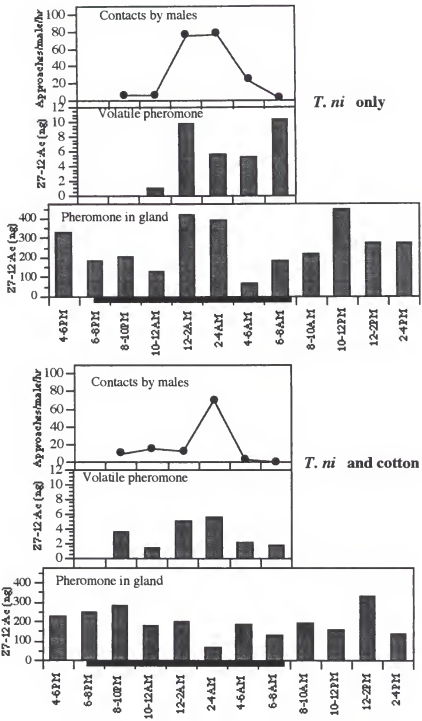

Figure 4.4. A comparison of the amount of pheromone present in glands, the amount released and the male response to volatiles emitted from *T. ni* females alone or on a bouquet of cotton. The study took place on October 15-16, 1994. The black bar represents the period of scotophase.

Males only responded to females on plants during the time period from 2:00-4:00 AM (Fig. 4.4). However, they responded equally to females without plants during the same time period and also during the previous time interval between 12:00 AM and 2:00 AM. Males were not active during dawn, although females were still releasing pheromone.

The following year, the amount of pheromone released by females with or without cotton was analyzed by volatile collection analysis beginning at the end of May through the middle of September, 1995. There was so much variation between trials, that each collection was evaluated separately (Fig. 4.5). The amount of pheromone released from females with or without cotton was about the same during May. However, during the months of June, a pattern was seen where more pheromone was released from females on cotton. If the 3 trials from June are combined, a significant difference is seen between females with or without cotton during the time periods from 12:00-2:00 A.M. (t (4) = 5.27, p =0.0045) and from 2:00-4:00 A.M. (t (4) = 3.87, p =0.018) (Fig. 4.6). From July through September, no consistent pattern between pheromone release on or off plants was observed. The time periods of release, the amount of pheromone released and relative amounts of pheromone released from females on or off cotton shifted with no apparent pattern.

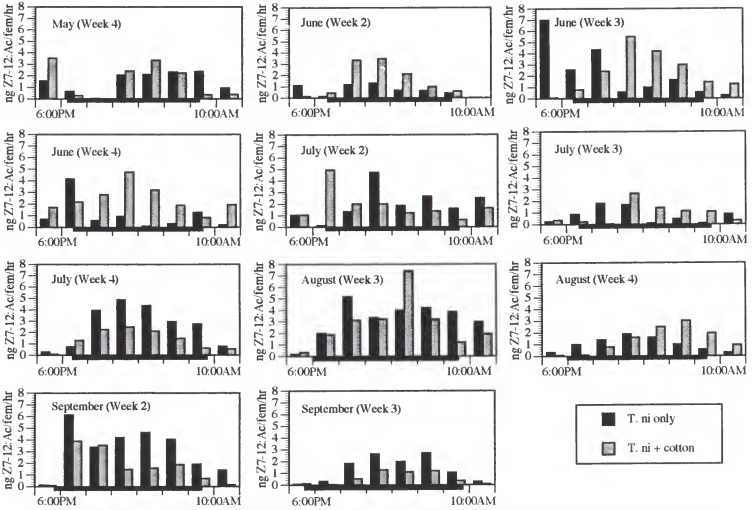

Figure 4.4. The amount of pheromone released from virgin females of *T. ni* from volatile collection analysis. Females were held alone or with a bouquet of cotton foliage. Collections took place from May to September of 1995. Dark bars on x-axis indicate the period of scotophase.

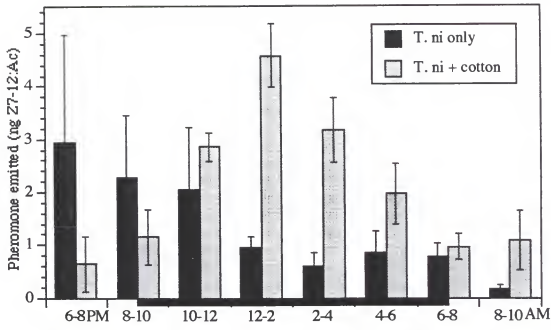

Figure 4.5. The amount of pheromone released from virgin females of *T. ni* during volatile collection analysis during June, 1995. Females were held alone or with a bouquet of cotton stems. The dark bar indicates the period of the scotophase (Bars indicate SEM, n=3).

Discussion

Though at times, there appeared to be an effect on pheromone production and release from the presence of host plants, the results were so inconsistent that no conclusion could be drawn. However, the amount of pheromone in glands was related to the amount of pheromone released. In previous studies where the amount of pheromone in the glands of *T. ni* was correlated to pheromone release, a similar pattern was seen where amounts in the gland decreased as the volatile release peaked (Hunt and Haynes, 1990). Hunt and Haynes (1990) evaluated volatile pheromone release during 24 hours, and they showed that pheromone was released throughout the 24 hour period. This could be due to the method they used for collecting volatiles where females were handled and placed in a special female

holder and the pheromone gland forcibly everted and placed in a glass tube for 10 minutes. The insects were also held under artificial conditions. Automated volatile collections systems, such as the one used in this study, probably reflect the normal behavior and physiological responses of females much better than rough handling techniques, since the insect is left completely undisturbed.

In the early summer, there was a difference in both the amount and time of pheromone release. Throughout the remainder of the summer, a consistent effect was not seen. This study needs to be repeated over several summers to determine if a seasonal host plant effect really exists. It would also be useful to use wild insects or insects held for only a few generations in colony to better reflect natural conditions. There are many uncontrolled environmental factors that could be affecting this study such as temperature, humidity, light intensity and atmospheric conditions. Each of these environmental factors could be affecting either the moths, the plants or both, making for a complex system, and hence the great deal of variation.

CHAPTER 5
THE EFFECT OF AGING AND CROWDING ON SEX PHEROMONE PRODUCTION AND OVIPOSITION IN VIRGIN FEMALES

Introduction

Mating brings about the termination of calling behavior or production of pheromone or both, in several species of Lepidoptera as well as the stimulation of oviposition. For example, Truman and Riddiford (1971) demonstrated that a successful mating by *Hyalophora cecropia* stimulated oviposition and terminated calling behavior. Females that received a spermatophore from a castrated male were not affected, suggesting that a factor associated with the sperm was responsible for this change from virgin to mated behavior. In subsequent studies, it was found that a successful mating stimulated the bursa to produce a blood-borne factor that triggered termination of calling and stimulated oviposition (Riddiford and Ashenhurst, 1973). Females of *Manduca sexta* displayed a short-term mated behavior after receiving a spermatophore from a castrated male. This behavior may have been caused by stretching of the bursa copulatrix, but sperm or testicular fluids had to be present to maintain this effect (Sasaki and Riddiford, 1984). Mating resulted in a decrease in sex pheromone production and increased oviposition in *Platynota stultana*, the omnivorous leafroller moth (Webster and Cardé, 1984) and also in *Lymantria dispar*, the gypsy moth (Giebultowicz et al., 1990). After mating, there is a rapid decrease in the pheromone titer in the pheromone glands of the females of *H. zea* (Raina et al., 1986).

This results from the action of peptides produced in the male accessory glands that are transferred to the female during mating (Raina, 1989).

Virgin gypsy moths produce less pheromone and increase oviposition as they senesce (Giebultowicz et al., 1990). Similarly, the amount of pheromone in the glands of virgins of *H. zea* declines with increasing age (Raina et al., 1986) and is accompanied by an increase in oviposition (Teal et al., 1990). Extracts of the bursa copulatrix, ovaries and hemolymph of aging virgins inhibited the action of PBAN when injected into young virgins (Teal et al., 1990). Although females of *H. zea* injected with brain homogenates produced less pheromone as they aged (Teal et al., 1990), it is unclear if this occurs under natural conditions and if so, if it is correlated with increased deposition of eggs by virgin females. This study was designed to determine the amount of pheromone produced and the pattern of oviposition by aging virgin females of *H. zea* without the use of an invasive technique. These results were compared with studies using mated females.

Gravid females of some species of Lepidoptera can detect the presence of conspecific eggs. For example, mated females of *Pieris brassicae*, *Pieris rapae* and *Danaus plexippus* use several cues to limit egg overload on host plants (Rothschild and Schoonhoven, 1977). Although oviposition deterrence is the most widespread response for mated females encountering other eggs (Thompson and Pellmyr, 1991), the presence of conspecific eggs may have no effect on oviposition preference, as is the case with mated females of the diamondback moth, *Plutella xylostella* (Groeters et al., 1992) or may even stimulate oviposition. For example, Navasero and Ramaswamy (1993) reported that the presence of conspecific eggs stimulated oviposition in mated females of *Heliothis virescens*.

During the study in the Chapter 5, where virgin females of *H. zea* were shown to oviposit increasing numbers of eggs as they senesced, it was noted that the insects routinely held in groups in 4-L cages, appeared to deposit more eggs per female than in the

smaller cages in which females were housed individually during the study. The experiments in this chapter were designed to determine if crowding or the presence of conspecific virgin females of *H. zea*, did in fact stimulate an increase in oviposition. It was also determined if the presence of conspecific females had any effect on the amount pheromone produced.

After the initial studies on the effect of crowding were conducted on *H. zea*, two other noctuid moth species, the cabbage looper, *T. ni* and the tobacco budworm, *H. virescens*, were also evaluated to determine if the presence of conspecific females affected their oviposition rates. Different combinations of species were placed together to find if interspecific females had the same effect as conspecific females.

Materials and Methods

Pupae of *H. zea*, *H. virescens* and *T. ni* were obtained from laboratory colonies maintained at the Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA-ARS, in Gainesville, FL. After the *H. zea* pupae were sexed, males and females were kept in separate cages in different incubators. Adult females were used immediately after eclosion in assays, whereas males were held separately for 24 hours after eclosion before being used for mating. The 3 different species of moths were kept apart and held in separate incubators, but under similar conditions.

Daily Rates of Egg Deposition by *H. zea*

Newly emerged virgin females of *H. zea* were placed singly in cylindrical, wire (0.6-cm mesh) cages (9.5 by 11.5 cm) lined with filter paper (Whatman no. 1) and having polystyrene petri plates (100 by 15 mm) for tops and bottoms. The cages were kept in a

growth chamber at 25 ± 2°C, 65 ± 3% RH with a photoperiod of 12:12 (L:D) hours. Each female was provided with a cotton wick soaked in 5% sucrose solution. The eggs laid by each female were counted every 24 hours. Another group of newly emerged females was treated in the same manner as virgin females except that a 1-day-old male was added to the cage on the first photophase after emergence. The male was removed after 24 hours. At the end of the experiment, the bursa copulatrix from females exposed to males was removed and dissected to determine if a spermatophore were present. Only those females found to contain a spermatophore were considered to have mated and were included in the study.

Determination of Pheromone Titer

The amount of pheromone present in pheromone glands of virgin and mated females of *H. zea* was evaluated from 1 day after eclosion to 15 days after eclosion. These females were kept under the same conditions as the females used for the egg deposition study. In preliminary studies, ovipositor extracts were obtained from 2-day-old females sampled at 1-hour intervals throughout the scotophase and analyzed by capillary gas chromatography. It was determined that the peak pheromone production occurred in the 5th hour of the scotophase (data not shown). These results are similar to those previously reported (Raina et al., 1986). Therefore, all insects were sampled during the 5th hour of the scotophase. Extracts of the pheromone gland were prepared by excising the terminal abdominal segments, which contain the pheromone gland, and placing the tissue in 10 μl of hexane containing 10 ng each of internal standards (octadecane and nonadecane). The major pheromone component of *H. zea*, (Z)-11-hexadecenal (Z11-16:AL) (Klun et al., 1980), was used to assess the amount of pheromone present in extracts. Extracts were analyzed with Hewlett-Packard 5890[®] gas chromatographs equipped with split/splitless

capillary injectors, flame ionization detectors, and fused silica columns (30 m by 0.25 mm [ID]) SPB1® (Supelco, equivalent to methyl silicone) and Supelcowax 10® (Supelco, equivalent to carbowax), under the following conditions: initial temperature = 60°C, injector operated in splitless mode for 0.5 min, oven temperature increased by 30°C per minute after 1 min, final temperature = 200°C, helium carrier gas at a linear flow velocity of 18cm/s. A Nelson Analytical 3000® (Cupertino, CA) data system was used to collect and reduce the data (Teal et al., 1989).

Effect of Presence of Conspecific Females on Egg Deposition and Pheromone Production

Newly emerged virgin females of *H. zea* were placed in cages and kept under the same conditions as described earlier for single insects, except that 1, 2, 5, or 10 females were held in each cage. The pheromone titer was assessed for 2-day-old females caged singly, or in cages containing 2 or 5 females. The eggs laid each day by females in cages containing 1, 2, 5, or 10, were counted every 24 hours for 8 days.

Effect of Interspecific Presence on Oviposition

Newly emerged virgin females were placed in cages and conditions as described earlier for the *H. zea* oviposition study in these arrangements: 1) one female per cage of *H. zea*, *H. virescens* or*T. ni*. ; 2) conspecific pairs of females of all three species; 3) one *H. zea* with one*H. virescens* ; 4) one*H. zea* with one*T. ni* ; 5) one *H. virescens* with one *T. ni* . In this way, each species was evaluated alone, with one other conspecific female and in each combination of interspecific pairs for all 3 species. Eggs were counted every 24 hours for 5 days at which time paper liners, petri plates and sugar water wicks were replaced. At the time this experiment was performed, the sucrose solution was decreased

from 5% used in the first oviposition experiments, to 2.5% due to mold problems in the main colonies.

Data were analyzed using one-way ANOVA, *t*-test, Fisher's LSD, Barlett's test and linear regression with Statview™ (Brainpower, 1985) and Instat® (Graphpad, 1993). Variability was reported as standard error of the mean.

Results

Effect of Aging on Pheromone Production and Egg Deposition in *H. zea*

The amount of pheromone (Z11-16:AL) present in extracts from virgin females of *H. zea* decreased steadily during the first 6 days after eclosion. However, the amount of pheromone increased after 7 days to levels half that present in extracts of 1-day-old females. The amount of pheromone then decreased between days 14 and 15 (Figure 5.1). Egg deposition rates were low for young females and gradually increased as the females aged (Figure 5.1). For the first 7 days, pheromone titer and the number of eggs laid were inversely correlated ($r = -0.898$). The number of eggs laid per day increased as pheromone titers dropped. However, there was no correlation between pheromone titer and oviposition rate for days 8-15 ($r = 0.257$).

The amount of pheromone present in extracts from mated females dropped significantly the same night as mating occurred (second scotophase) and remained low for the duration of the experiment (Figure 5.2). The rate of egg deposition increased greatly during the 24 hour period following mating and peaked on the third photophase (Figure 5.2). The number of eggs laid declined steadily until it reached levels not significantly

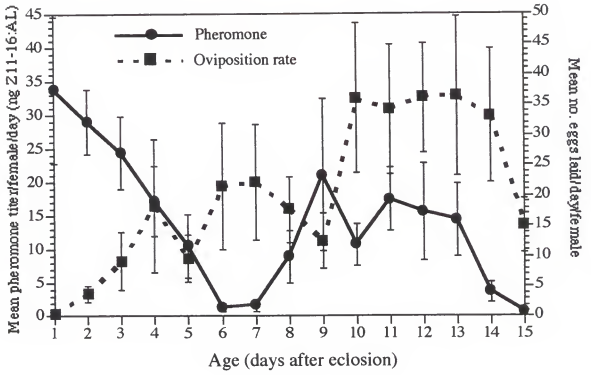

Figure 5.1. The amount of (Z)-11-hexadecenal extracted from the terminal abdominal segments (n = 10; bars ± SE) and the average number of eggs deposited each day (n = 28; bars ± SE) by virgin *H. zea* females held singly from 1 to 15 days after eclosion .

different (F (1, 44) =1.745, $p > 0.01$) from those of virgin females around the 10th day of the experiment (compare Fig. 5.1 with 5.2).

Virgin females laid far fewer eggs than mated females and the life-time patterns of oviposition were different. Mated insects laid more than 56% of their total eggs within 3 days (2 to 4 days after eclosion) and laid over 78% in the 1st week. Virgin insects laid more than 62% of eggs in the last 6 days of the experiment and only 10% in the first 3 days and 27% in the 1st week.

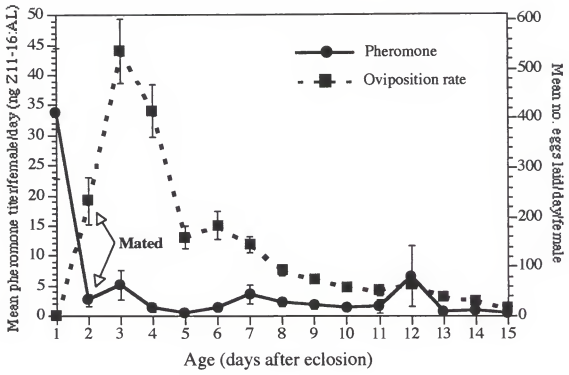

Figure 5.2. The amount of (Z)-11-hexadecenal extracted from the terminal abdominal segments (n = 10; bars ± SE) and the average number of eggs deposited each day (n = 22; bars ± SE) by mated *H. zea* females held singly from 1 to 15 days after eclosion .

Effect of Presence of Conspecific Females on Egg Deposition and Pheromone Production

The oviposition rate of single virgin females increased dramatically when one other virgin female was present. The average number of eggs laid per female over 8 days by

single females was 153.4 ± 52.56, and the average per female when two females were caged together was 600.2 ± 119.1. The average total number of eggs laid per female for five insects to a cage was 549.6 ± 105.19 and for 10 insects to a cage was 535.2 ± 37.37. Groups of 2, 5, or 10 females all showed significantly greater egg deposition per female than single females (F (3, 36) = 5.77, $p \leq 0.005$). The totals of eggs laid when either 2, 5, or 10 females were in a cage were not statistically different (F (2, 27) = 0.131, $p >$ 0.25). The daily oviposition rates of females from groups of 2, 5, or 10 were within the same range, whereas single females laid far fewer eggs after the 3rd day than those virgins kept in groups (Fig. 5.3). By the 2nd day after eclosion, single females were laying significantly fewer eggs than two females per cage (t (18) = 1.843, 0.1 $p \leq 0.05$) or five females per cage (t (18) = 2.636, 0.1 $p \leq 0.01$).

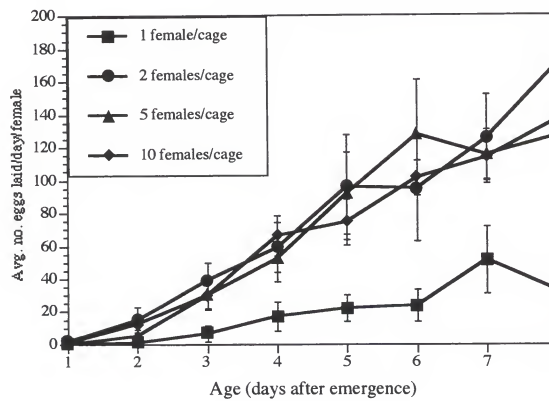

Figure 5.3. The average number of eggs laid each day by virgin *H. zea* females from 1 to 8 days after eclosion. Insects were held 1, 2, 5 or 10 to a cage (n = 10; bars ± SE).

Based on this difference, the pheromone titer was assessed for 2-day-old virgin females held singly, two per cage, and five per cage during the peak of pheromone production (5th hour of scotophase). No difference was found in the amount of pheromone among these groups (F (4, 110) = 1.09, $p< 0.25$). The average quantity of pheromone of single females was 54.22 ± 6.93 ng Z11-16:AL, whereas the average per female of two females was 59.98 ± 7.46 ng Z11-16:AL, and that of five females per cage averaged 47.29 ± 4.92 ng Z11-16:AL.

Effect of Interspecific Presence on Oviposition

All females held individually laid the about the same number of eggs per female (F (2, 30) = 1.104, p= 0.345) (Fig. 5.4). Statistical analysis using Fisher's LSD showed that *H. zea* pairs were different from *H. zea* single females, but *H. virescens* or*T. ni* females who were held individually or in pairs had no difference in the number of eggs that were deposited (Fig. 5.4). The number of eggs laid by *H. virescens* held in pairs, however, were no different from the eggs laid by *H. zea* pairs. The lower concentration of sucrose solution offered to the insects did not appear to have any significant effect on oviposition, from comparison with the results from the *H. zea* crowding study. *H. zea* females, which were held individually in the first study and were given 5% sucrose, laid an average 52.6 ± 18.36 eggs over a 5 day period, and the *H. zea* females in this study held individually and given 2.5% sucrose, laid an average of 57.6 ± 25.6 eggs over the same time period. Pairs of *H. zea* females in the first study deposited 180.7 ± 46.21 eggs per female. The number deposited by females in the interspecific oviposition study laid 144.32 ± 44.39 eggs per female. There was no significant difference in females held in pairs who received 2.5% or 5% sucrose (t (21) = 0.565, p = 0.578) or females held singly (t (20) = 0.159, p = 0.876).

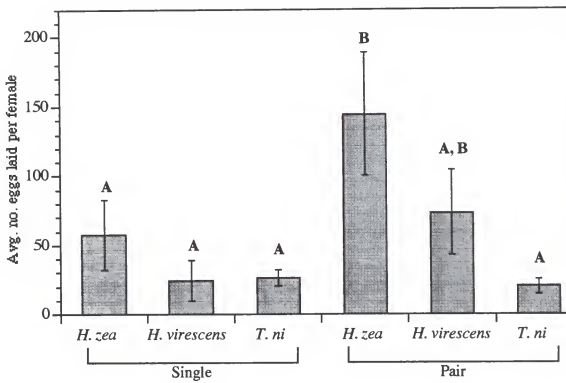

Figure 5.4. The cumulative number of eggs deposited per female over 5 days by *H. zea*, *H. virescens* or*T. ni* held in cages individually or in pairs (n = 10; bars ± SE).

There was a significant difference in the number of eggs deposited between the different interspecific and conspecific pairs of females (F (5, 54) = 2.52, p= 0.040) (Fig. 5.5). However, the variation among different groups was significantly greater than expected by chance as shown by Barlett's test for homogeneity of variances (Barlett stat=57.81, $p < 0.0001$). Therefore the data were logrithmically transformed and a t- test was used to analyze the data. Pairs of *H. zea* females did not lay more eggs than either

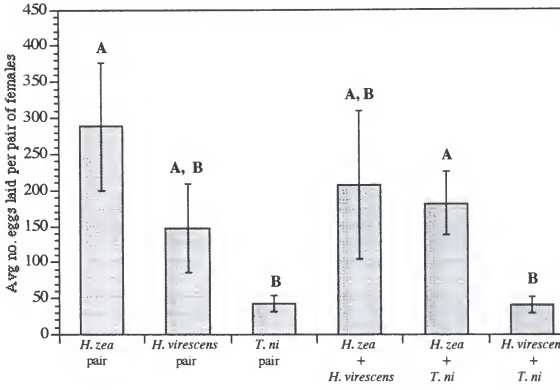

Figure 5.5. The cumulative number of eggs deposited by 2 females held together in conspecific or interspecific pairs for 5 days (n = 10; bars ± SE).

pairs of *H. virescens* (t (19) = 1.61, p= 0.124) or an interspecific pair of *H. zea* and *H. virescens* (t (19) = 1.72, p= 0.102) (Fig. 5.5). The interspecific combination of *H. zea* and *T. ni* caused an increase in the number of eggs laid compared to conspecific pairs of *T. ni* (t (17) =2.5, p= 0.023). The other combinations of non-*H. zea* interspecific pairs

did not differ from *H. virescens* or *T. ni* conspecific females caged together. Therefore, only *H. zea* females showed an increase in oviposition when held with other females of their own species (Fig. 5.4) and were the only species to cause a significant increase in oviposition when held with interspecific females (Fig. 5.5).

Discussion

These results show that the relationship between pheromone production and oviposition is inversely correlated for both virgin and mated adult females. As pheromone titer decreases, the rate of oviposition increases. This pattern, the shift from virgin to mated behavior, occurs immediately after mating takes place. Yet, as virgin females age, this change in behavior also occurs. The decline in the pheromone titer of virgin females is probably caused by the activity of an inhibitory factor in the bursa copulatrix and ovaries. Injection of one female equivalent of extract of the bursa copulatrix from 5-day-old virgin females into younger (3-day-old) females along with brain-subesophageal homogenates resulted in the production of less pheromone when compared with injection of brain homogenates alone (Teal et al., 1990). The correlation between the increased egg deposition and decreased pheromone titer over the first week suggests that oviposition in virgin females may be influenced by the same factor which inhibits the action of PBAN. The similarity between mated and aging virgin females suggests that mechanisms controlling the change from virgin to mated behavior may be related.

Based on data obtained from the first 7 days, it was hypothesized that oviposition rates would increase and pheromone titer decrease as virgin females age. Although oviposition rates continued to increase over the second week, the amount of pheromone showed an accompanying increase. However, the amounts were not as great so those found during the second night and declined to very low levels on the last two nights of the

study. It is not known if the inhibitory factor associated with the bursa copulatrix and ovaries is produced continuously, therefore, it cannot be explained why the amount of pheromone increased during the second week.

A similar pattern of declining pheromone titer and increased oviposition with age was shown in gypsy moth, *L. dispar*, although it does not appear to be associated with the bursa copulatrix or the spermatheca, as removal of these organs did not prevent this pattern (Giebultowicz et al., 1990). The study involving gypsy moths lasted for one week, so it is not known if gypsy moths begin a second period of pheromone production, as does *H. zea*.

Raina (1989) showed that pheromone titers of mated *H. zea* females decreased significantly within less than one hour following mating and within two hours decreased to almost nothing. In this study, mating also resulted in a precipitous decline in the amount of pheromone on the night of mating. However, the amount remained low for the remainder of the study. These data do not coincide with those reported by Raina et al. (1986), which showed a partial recovery in the pheromone titer (approximately one third that of unmated 2-day-old females) of mated females during the third to fifth scotophase. However, in gypsy moths the pheromone titer drops significantly after mating and remains low for five days (Giebultowicz et al., 1990).

The study presented here demonstrated how aging virgin females show a correlation in the production of pheromone and the rate of oviposition, similar to that seen in younger mated females. The next chapter will explore the role of the bursa copulatrix in aging virgin females and determine if bursa-borne factors regulate both pheromone decline and oviposition rate increase as virgins age.

This study also indicates that the presence of conspecific virgin females of *H. zea* stimulated oviposition and that only one other female needed to be present to elicit this response. Adding five or 10 females to a cage does not increase oviposition rates any more

than two insects, and surprisingly, although 10 females to a cage produced considerable crowding, the rate of oviposition did not decline, but remained the same as in groups of two or five females. Conspecific presence greatly accelerates the rate of egg deposition seen in single aging virgins. However, a pronounced effect is seen as early as two days after eclosion. However, pheromone titer is not affected at two days after eclosion by the presence of conspecific virgin females. Conspecific eggs or oviposition marking pheromones from mated lepidopteran females generally produce a deterrent effect when encountered by another female (Thompson and Pellmyr, 1991), although conspecific egg presence may have no effect (Groeters et al., 1992) or may result in stimulation of oviposition (Navasero and Ramaswamy, 1993). Navasero and Ramaswamy (1993) reported an increase in oviposition of *H. virescens* mated females on cotton leaf terminals, which they attributed to the presence of conspecific eggs. Unfortunately, they did not use control treatments which could have confirmed if an increase in oviposition really occurred.

Although *H. zea* females are stimulated to oviposit more eggs when another female is present, females of *H. virescens* or *T. ni* did not show any significant increase in oviposition when held in conspecific pairs. When a female of either of these species was caged with an *H. zea* female, however, there was an increase in the number of eggs laid than those deposited by either *H. virescens* or *T. ni* conspecific pairs. Although, it was not determined which species the eggs came from in the interspecific pairs during this study, the data suggest that the *H. zea* females were stimulated to lay more eggs when conspecific or interspecific females were present.

Avoidance mechanisms, which appear to be the general case for most gravid lepidopteran females, could be beneficial for a mated female in the placement of fertile eggs to provide an ample food supply or avoid cannibalism for her progeny. However, little is known about how oviposition mechanisms operate in virgin females and why virgin females laying unfertilized eggs increase the oviposition rate when other virgin females are

present. The data show such a significant increase in oviposition when other females are present, particularly conspecific virgin females, that it suggests that an oviposition stimulating pheromone may well be present.

CHAPTER 6
THE ROLE OF THE BURSA COPULATRIX IN THE SUPPRESSION OF PHEROMONE PRODUCTION AND IN THE STIMULATION OF OVIPOSITION IN AGING VIRGIN FEMALES

Introduction

In the previous chapter, a correlation was seen between the natural decline of pheromone production and an increase in the deposition of eggs by aging virgin females of *H. zea*. As previously discussed, extracts of the bursa copulatrix inhibit the action of PBAN (Teal et al., 1990). Pheromonostatic material was found in aqueous extracts of the bursa copulatrix of *H. zea*. Extracts of the ovaries and hemolymph also contained some pheromone suppressing activity. When bursa extracts were extracted with organic solvents such as pentane, hexane or iso-octane, the activity of the aqueous phase was not diminished and no activity was found associated with organic solvents (Teal et. al, 1990). This would eliminate juvenile hormone from being the compound of interest, since it would be extracted in these solvents. Teal et al. (1990) also examined the possibility that the other major group of insect hormones, the ecdysteroids, might be candidates for the pheromonostatic compound. However, injection of various amounts of either ecdysone or 20-hydroxyecdysone had no effect on pheromone production.

The effect of the pheromonostatic bursa factor, pheromone biosynthesis suppression factor (PBSF), increases in a linear fashion and completely suppresses pheromone production at a dose of 2 female equivalents (FE). Injection of PBSF 30 or 60 minutes prior to injection of PBAN completely eliminates pheromone production (Teal et al., 1990). If PBSF is injected 30 minutes after PBAN has already been injected, and the

amount of pheromone assessed one hour later, the same amount of pheromone is produced as if the females had been incubated for 30 minutes after injection of only PBAN.

Giebultowicz et al. (1990) reported a response in virgins of gypsy moths similar to that seen in *H. zea*, with decreased pheromone production and increased egg deposition being associated with age. However, removal of the bursa copulatrix from gypsy moth females had no effect on this response. In order to establish if the bursa is directly involved in stimulating an increase in oviposition in *H. zea*, which was implied by the correlation of pheromone suppression and increased oviposition with senescence, an implant experiment was devised where the insect's own bursa was left intact and newly emerged females were implanted with additional bursa tissue from aging donor insects. An implant surgery was used because the bursa copulatrix of *H. zea* is a large organ, extending almost the entire length of the abdomen (see photo in Fig. 6.1) and is surrounded by ovarian tissue making removal of the bursa, without disruption to the internal organs of the insect, nearly impossible. Another goal of this study was to purify PBSF from the bursa and determine if the same compound(s) do in fact regulate pheromone suppression and oviposition stimulation.

Materials and Methods

Pupae of *H. zea* were obtained from a laboratory colony maintained at the Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA-ARS, in Gainesville, FL. The pupae were separated by sex and after eclosion, the females were held in 4-l cages and provided with cotton wicks soaked with 5% sucrose solution. The insects were held at 25 ± 2 °C, 65 ± 3% relative humidity with a 14:10 (L:D) photoperiod.

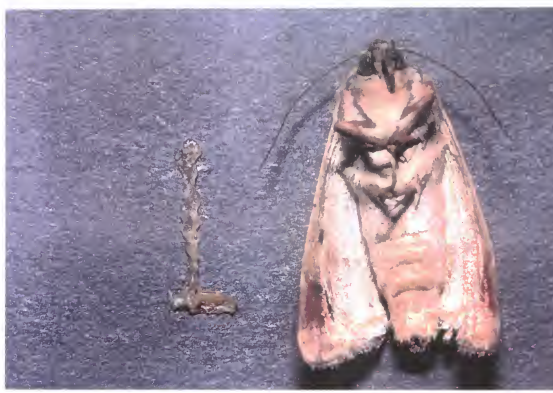

Figure 6.1. Photograph of a female of *H. zea* next to a bursa copulatrix. Note the size of the bursa relative to that of the abdomen of the insect.

Virgin females were used for pheromone suppression bioassays 1 and 2 days after eclosion during the 7th-8th hour of the photophase and newly emerged females were used for oviposition studies.

The Effect of Implantation of the Bursa on Oviposition

The bursa copulatrix was removed from either newly emerged or 5-day-old donor females. Because of the great size of the bursa copulatrix, the corpus bursa was separated from the cervix bursa (see Fig. 6.2 for structure). The cervix bursa was discarded and the corpus bursa was placed in sterile Grace's medium containing 0.005 mg/ml of gentamicin and a few crystals of phenylthiurea (PTU) to prevent oxidation and melanization of tissue.

Newly emerged recipient insects were implanted with the corpus bursae. The lateral side of the abdomen of the recipient was swabbed with 70% ethyl alcohol:water, followed by Grace's medium. A small lateral incision was made between the 3rd and 4th abdominal sclerites, and the corpus bursa was slipped into the opening with a small probe. The wound was pressed and sealed. No adhesive material was necessary. It was important to use newly emerged females as recipients, since the ovaries fill with eggs quickly, making the closing of the incision or the implantation of the bursa much more difficult. Recipient insects received a bursa from either a young female (newly emerged) or a 5-day-old female. Sham operated females were used for controls and had the same procedure done but no bursa inserted.

After implantation surgery, each insect was individually placed in cylindrical, wire (0.6-cm mesh) cages (9.5 by 11.5 cm) lined with filter paper (Whatman no. 1) and having polystyrene petri plates (100 by 15 mm) for tops and bottoms. The cages were kept in a growth chamber at 25 ± 2°C, 65 ± 3% RH with a photoperiod of 14:10 (L:D) hours. Each

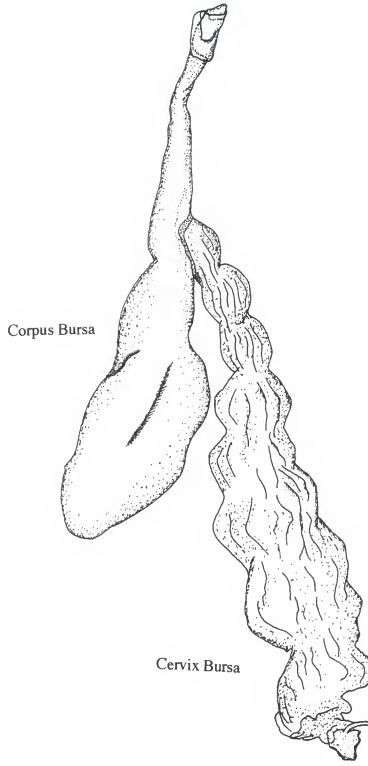

Figure 6.2. The bursa copulatrix of *H. zea*, showing the corpus bursa and the cervix bursa (Drawing provided courtesy of O. Molina).

female was provided with a cotton wick soaked in 5% sucrose solution. The total number eggs laid by each female were counted every 24 hours for 3 days.

Purification of PBSF from the Bursa Copulatrix

During the purification procedures, the pheromonostatic activity of fractions was tested in the following manner. The fractions were alliquoted and lyophilized to dryness. The dried fractions were reconstituted in physiological saline (Christensen et al., 1991), so that each female was injected with 20µl containing 5 pmol of PBAN. A saline blank and a PBAN positive control was included in each assay. Insects were treated with 3FE of the test fraction unless otherwise indicated. The insects were allowed to incubate for 1 hour after injection and then the pheromone gland was excised and extracted. The amount of pheromone in extracts was determined as previously described in Chapter 2.

The bursa copulatrix were removed from 6 and 7-day-old females, when the amount of pheromone produced under natural conditions is the lowest (Fig. 5.1). The bursa were placed on dry ice immediately after removal. Groups of bursa were stored at -60°C until 1000 bursa were obtained. At this point, the bursa were lyophilized for 4 hours. The bursa were then ground into a fine powder in a commercial coffee bean grinder. The powdered bursa were extracted 3 times in 20 ml of ethyl acetate to remove apolar to moderately polar lipiphilic compounds. Ethyl acetate extracts were filtered using Whatman no. 1 filter paper. The bursa powder was removed from the filter paper and was then suspended in 20 ml of cold H_2O containing 0.1% trifluoroacetic acid (V:V) (0.1%TFA), mixed, and sonicated for 15 minutes in ice water. The suspension was centrifuged at 6 x g for 15 minutes, and the supernatant collected. The pellet was resuspended in 0.1% TFA, sonicated, centrifuged and the supernatant collected and combined. The combined

supernatant was further filtered through glass wool. This supernatant was referred to as the crude aqueous bursa extract. The crude extract was tested for pheromonostatic activity.

A Bakerbond® octadecyl (C18) narrow-pore (60 Å), reverse-phase solid phase extraction (spe) column (6 ml, 500mg, 40μm) was preconditioned as follows. The column was washed with 12 ml of 0.1% TFA, followed by 1ml of a solution containing 5 mg of bovine serum albumin (BSA) in 0.1% TFA to bind active sites on the column. Then the column was washed thoroughly with 0.1% TFA to remove unbound BSA, followed by 12 ml of 80% acetonitrile:H_20 containing 0.1% TFA, followed by 20 ml of 0.1% TFA in water. After this preconditioning, 100 FE of the crude bursa extract was loaded, eluted and collected. The column was washed with 6 ml of 0.1% TFA and this was combined with the void and tested. The column was then eluted with 12 ml 80% acetonitrile (0.1%TFA) and this fraction was collected and tested.

The active material from the C18 spe step was applied to a Bakerbond® butyl (C4) wide-pore (250 Å) (6 ml, 500mg, 40μm) spe column. The column was prepared and preconditioned with 12 ml of acetonitrile (containing 0.1% TFA) followed by 12 ml of 0.2M acetic acid. The active fraction from the C18, was loaded and eluted and then the column was rinsed with 12 ml of 0.2M acetic acid, which was combined with the void volume and tested. Then the column was eluted with 80% acetonitrile:H_20 (0.1% TFA) and this fraction was tested for activity.

A Bakerbond® silica spe column was prepared (6 ml, 500mg, 40μm APD, 60 Å) and preconditioned with 12 ml of water buffered to a pH of 9.5 with a solution of 0.67M NH_4OH (containing 20g/l EDTA). The 0.67M NH_4OH solution was added to the acidic C4 void until the pH was brought to 9.5 and then it was applied to the column. The column was then washed with 12 ml of buffered water (pH 9.5) and this was combined

with the void volume and tested. The column was then eluted with 12 ml of H_20 (pH 6) followed by 12 ml of 0.2M acetic acid. Both the H_20 and 0.2M acetic acid fractions were collected and assessed for biological activity separately.

The active material from silica was chromatographed using both cationic and anionic ion exchange chromatography. First, the active fraction from the silica column was placed on a Bakerbond® PEI (phenylethyleneiminesilane) weak anion column (6 ml, 500mg, 40µm APD, 240 Å) which had been preconditioned with NH_4OH buffered water (pH 9.5). The void volume was collected, the column rinsed with 12 ml buffered (pH 9.5) water and this combined with the void volume. The column was then eluted with H_20 (pH 6), followed by 0.1% TFA. All fractions were tested for activity. For cation exchange chromatography, a Bakerbond® sulfonic acid (strong cation) column (6 ml, 500mg, 40µm APD, 240 Å) was preconditioned with 12 ml 0.1% TFA. The active material from the silica column was acidified to pH 2.8 with 0.1% TFA. This material was applied to and eluted from the sulfonic column followed by 12 ml of 0.1% TFA (pH 2.8). The 2 were combined and tested. The column was then eluted with 12 ml of H_20 (pH 6.0) and then 12 ml of buffered (NH_4OH to pH 9.5) water. Each fraction was tested for activity.

Effect of PBSF on Oviposition

Females that were 1-day-old were injected either once or twice with 10µl of saline containing 5 FE of partially purified PBSF obtained form the silica column separation. Control insects were injected with saline only. If 2 injections were given, the second injection took place 24 hours following the first injection. Those insects receiving only 1 injection of PBSF, were given an injection of saline when the second group received their second dose. The females were placed in small wire cages as described in the section on

implants. Seventy-two hours from the initial injection, the total number of eggs was counted.

Data were analyzed using one-way ANOVA, Mann-Whitney test and *t* -test with InStat® (Graphpad, 1993). Variability was reported as standard error of the mean.

Results

The Effect of Implantation of the Bursa on Oviposition

Sham-operated and females that were implanted with a corpus bursa from young donors, laid the same number of eggs (t (31) = 0.149, p =.0.88), whereas females who received a bursa corpus from older females laid more eggs (t (31) =2.35, p = 0.026) (Fig. 6.3). Due to the high degree of variation, especially in the group of females receiving bursa from older donors, a nonparametric test was also used to assess the difference between the number of eggs laid from females with old versus young bursa and it showed that the difference between the two groups was extremely significant (Mann-Whitney U = 50.5, p = 0.002). Only the bursa implanted from aging females caused a marked increase in egg deposition.

Purification of PBSF from the Bursa Copulatrix

The first step in isolation of PBSF from the bursa was the development of a bioassay for use with fractions from chromatographic separations. This was crucial because the focus of this study was inhibition of pheromone production, rather than stimulation. Thus, a positive pheromonostatic result was indicated by production of

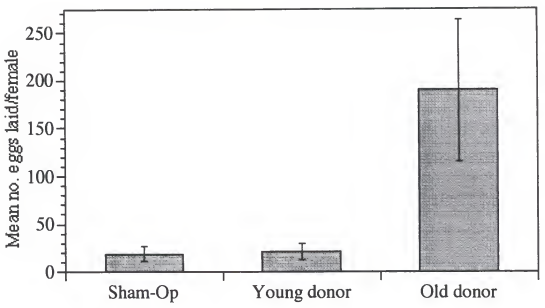

Figure 6.3. The total number of eggs laid after 72 hours by sham-operated *H. zea* females or females surgically implanted with a corpus bursa from either newly emerged or 5-day-old donor virgin females. Recipient insects were newly emerged virgins (Bars indicate SEM, n=15).

significantly less pheromone than was produced by females injected with only PBAN. In initial studies using crude aqueous homogenates of the bursa (see Teal et al., 1990), it was found that injection of 3 bursa equivalents (BE) along with 5 pmol of synthetic PBAN resulted in a highly significant inhibition of pheromone production when compared to females injected with only 5 pmol of synthetic PBAN. Therefore, this dose was selected for subsequent studies.

Initially, the lyophilized powdered bursa were subjected to extraction with an organic solvent of medium polarity (ethyl acetate) followed by extraction with acidified H_2O. Bioassays of these two extracts indicated that no inhibitory activity was associated with ethyl acetate extract, but the aqueous extract was found to induce the production of only 12% as much pheromone as was produced by females injected with PBAN. This

eliminated moderately polar and apolar lipids, like juvenile hormone, as potential candidates and resulted in substantial purification.

The first chromatographic separation was performed by applying the aqueous extract to a preconditioned narrow pore C18 spe column (Fig. 6.4). The void volume and aqueous fraction collected from this step had only 8% of the activity of PBAN controls, whereas the 80% acetonitrile fraction had 96% of the activity of the positive PBAN controls. Thus, PBSF was not retained on the C18 column. This indicated that the material was either polar or incapable of penetrating the narrow pore matrix. However, it did not exclude the possibility that the molecule was a large peptide or protein, because such molecules have low affinity for narrow pore C18 matrices (Schooley et al., 1989). To address this, the aqueous fractions from the C18 were applied to a preconditioned C4 spe column, known to effectively retain apolar peptides and proteins (Schooley et al., 1989). The combined void and aqueous wash fractions from this step induced less than 1% of the pheromonotropic activity found in positive controls, whereas the 80% acetonitrile fraction, which contained the apolar peptides and proteins, had 88% of the activity of PBAN controls.

All three fractions collected from the silica separation inhibited the production of pheromone when injected along with 5 pmol of PBAN (Fig. 6.5). However, when females were injected with either the basic (NH_4OH) and acidic (acetic acid) fractions, the general activity level of the insects was much reduced relative to control treated insects and some insects appeared to be paralyzed. A similar situation was noted when testing the eluants from the reversed phase columns and may reflect the inability to completely remove acidic or basic ion pair reagents when lyophilizing fractions. However, insects treated with the H_2O fraction from the silica spe separation, which contained only residual amount of ion pair reagent, did not suffer any adverse effects and behaved like

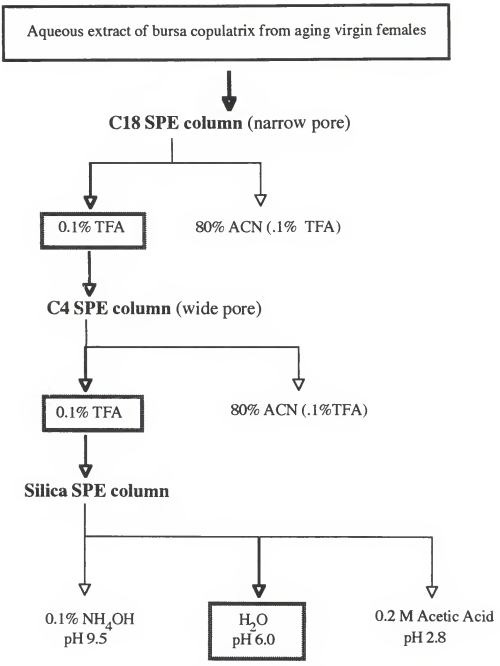

Figure 6.4. Purification procedure for the pheromonostatic factor in aqueous extracts of the bursa copulatrix. Active material was not retained though the 2 reversed phase spe steps, but was retained on the silica spe column and was eluted with H_2O. Boxes indicate that the fraction showed pheromonostatic activity in bioassays.

control treated insects. This fraction was chosen for ion exchange chromatographic studies, because it had no adverse effects on the insects, but still completely inhibited pheromone production.

An overview of the ion-exchange separations is given in Fig. 6.6. PBSF was not retained on the strong cation-exchange (sulfonic) column (Fig. 6.7). However, activity was retained on the PEI (weak anion) column and eluted in the H_20 fraction (Fig. 6.7). The insects injected with both of these fractions appeared and behaved like the insects injected with the H_20 fraction from silica.

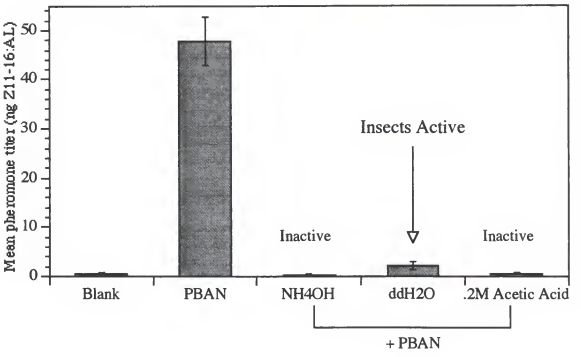

Figure 6.5. Amount of pheromone produced by insects injected with PBSF silica fractions containing 5 pmol of PBAN. The insects injected with the H_20 behaved normally (Bars indicate SEM, n=4).

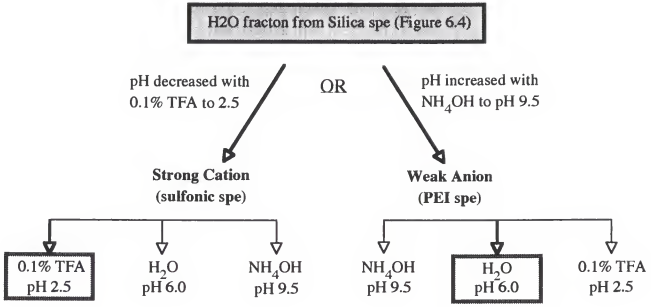

Figure 6.6. The active fraction from the initial separation was placed on either a sulfonic (strong cation) spe column or a PEI (weak anion) column. Pheromonostatic material was not retained on the sulfonic separation, and was found in the void. The pheromonostatic material was retained on the PEI column and was eluted with H_2O.

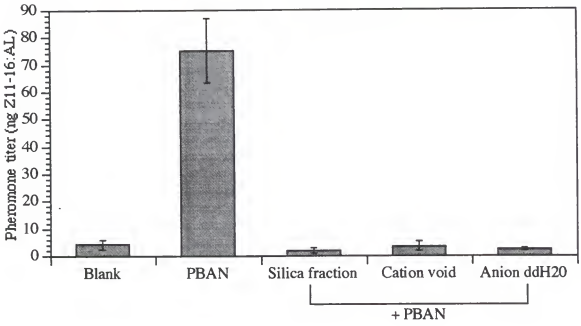

Figure 6.7. The pheromonostatic response of females of *H. zea* injected with fractions from ion-exchange separations compared to the active fraction from silica. Each spe fraction was injected with 5pmol of PBAN (Bars indicate SEM, n=4).

Effect of PBSF on Oviposition

Females injected twice in 24 hour intervals with 5 FE of the silica H_20 PBSF fraction laid significantly more eggs (> 10 times more) than control females (t (7) =5.99, p = 0.0005) (Fig. 6.8). The experiments lasted for 72 hours, and there was very low mortality, showing that the silica H_20 fraction was free of the toxic substances. Insects injected only once, laid almost 6 times as many eggs as controls (Fig 6.9). However, the variation was so great in the PBSF treated insects, that there was no significant difference with saline treated insects (t (7) =1.73 p = 0.127). Log-transformation of the data still

showed no significant difference between insects injected only once and saline controls (t (7) =1.88 p = 0.102). There was, however, no significant difference between insects injected either once or twice with the partially purified PBSF material (t (6) =1.01 p = 0.313). The large significant difference between controls and insects that were injected twice with PBSF indicates that the same fraction that suppresses PBAN, clearly stimulates oviposition.

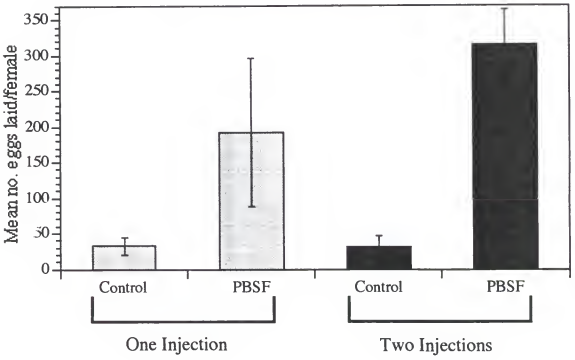

Figure 6.8. The number of eggs laid by females of *H. zea* after injection of the silica H_2O fraction. Females were either injected once with 5 FE or twice with 5 FE 24 hours apart (Bars indicate SEM, n=5)

Discussion

The results presented here strongly support the view that the naturally occurring decline in pheromone titer and the increase in oviposition seen in senescing virgins of *H. zea* is due to a factor present in the bursa copulatrix. Females implanted with a corpus bursa from aging females (5-day-old) were stimulated to lay far more eggs than sham-operated or females implanted with corpus bursa from young females. The bursa is likely secreting a substance that is causing this premature or mated-like oviposition response. The recipient insects were only 3-days-old at the end of the experiment, and would normally lay very few eggs at this age (Fig. 5.1). However, females implanted with the corpus bursa from 5-day-old females laid more eggs than the average virgin produced in the entire 15 day period of the study in Chapter 5. In fact, these females laid about half the number of eggs that mated females deposited within 3 days during peak egg production (Fig. 5.2). This indicates that the implantation of the copus bursa caused a behavioral and physiological shift from a "virgin" to "mated" condition similar to that which occurs after a successful mating.

Unknown compounds present in the aqueous fractions from the reversed phase separations, as well as in the acidic and basic fractions from the silica separation, had deleterious effects on the insects. The compounds responsible for this could have been naturally occurring. If these compounds were released from the bursa, the insects would have suffered negative effects when the corpus bursa was implanted. This was clearly not the case, because females who received implants survived the treatments and exhibited no adverse effects. The compounds could have been released when the bursa tissue was ground up, so that enzymes and other toxic substances that are usually segregated within the cells were liberated. Another possibility is that the deleterious compounds could have

resulted from chemical manipulation during sample preparations. One likely source of these compounds is the ion pair agents used (i.e. TFA, acetic acid or NH_4OH), because all are rather strong oxidizing or reducing agents which would be expected to cause significant physiological problems. Although these reagents were selected for their volatility, which should have allowed for their vaporization during lyophilization, the large volume of solvents used for separations, make total vaporization difficult and thus traces of the reagents were probably present in the samples injected into the insects. This hypothesis is supported to some degree by the fact that the neutral fraction (H_2O fraction) from the silica separation, had no deleterious effects on the insects while both the basic and acid fractions, which contained strong ionic reagents were effectively toxic.

The pheromonostatic material was retained on the silica column which had been conditioned and eluted with aqueous buffers. The use of aqueous solutions altered the retention characteristics of the silica gel. The silica matrix is so polar, that the water molecules of the buffers would effectively bind all active sites on the column. Therefore, the buffer acted as the separation media as it was bound to the column. The NH_4OH buffer that was used to precondition the silica column prior to application of PBSF, probably effectively converted the column into a weak ion exchange column from the NH_4^+ in solution. If this is so, then the elution of PBSF from this column with H_2O at pH 6.0, would indicate that the material is weakly acidic when in strongly basic solution. However, in neutral or slightly acidic solutions, the material lost its charge and was released from the matrix. This is supported by the results of the ion exchange experiments, because the material also showed the same characteristics when chromatographed at high pH using the anion exchange column. However, the material is not amphoteric, because it failed to bind to the cation exchange resin when applied at low pH. This suggests that PBSF may be a weak acid.

To date, compounds involved in induction of mated-behaviors in female insects, i.e. suppression of pheromone and stimulation of oviposition, are peptidic in nature. For example, the *H. zea* male-borne factor that is produced in the accessory glands, transferred to the female during mating shutting down pheromone production, is a 57 amino acid peptide (Kingan et al., 1995) and a neuropeptide recently isolated from the brains of female wax moths induces oviposition in crickets (Abdoun et al., 1995). These peptides have no homology. Results from this study indicate that the compounds responsible for both pheromonostatis and induction of oviposition in *H. zea*, are either the same or quite similar chemically, because of their chromatographic behavior. Although the isolation and identification of PBSF is not yet complete, from this study we have gained significant information about its chemical properties. We know that the material is: 1)not an apolor or moderately polar lipid, as it is insoluble in ethyl acetate; 2) is not an apolar polypeptide or protein because it is not retained on revered phase matrices; 3) is highly polar as it is soluble in H_2O; 4) is charged at high pH, suggesting that it is a weak acid. The failure of PBSF to fit neatly into any category of identified insect hormones has made its isolation very challenging.

The evolutionary implications of PBSF are also challenging. The existence of mated-behavior inducing substances from the standpoint of both males and mated females are useful. Females do not remate if they no longer behave as virgins, protecting the investment of the male. A mated female also benefits, especially among short -lived species, because she is able to lay eggs almost immediately after mating, does not invest energy in the production of pheromone and is not disturbed by males seeking mates when she is laying eggs. Aging virgin female moths are probably not very common in nature. Older virgin moths who do not produce much pheromone are advantageous to younger females because of lack of competition and to males who have a higher probability of reproducing if they mate with younger females. However, what benefits would belong to

the aging virgin female remains unknown. It is possible that PBSF actually acts in mated females and is released after stimulation from some other factor that is directly related to mating. As females age, PBSF may be released due to aging of the tissues or other physiological events.

CHAPTER 7
SUMMARY AND CONCLUSIONS

The goals of this research were to understand how endogenous and environmental factors regulate both the stimulation and suppression of pheromone production in virgin females of noctuid moths. The first component of pheromone regulation that was explored was endogenous stimulation, the first regulatory step for the production of pheromone. Structure-activity studies of PBAN and fragments of PBAN showed an interesting correlation of structure and function between PBAN and myotropic pyrokinin peptides (Nachman et al., 1986; Raina and Kempe, 1990). Both myotropic peptides and PBAN required the same minimal sequence to produce their respective physiological functions. From these studies, it was obvious that there could be cross-activity between these two groups of peptides and there was (Fonagy et al., 1992b; Kuniyoshi et al., 1992). No comprehensive study had been completed, until now, to demonstrate the importance of such variables as the size, substitution of amino acids in the active core region of the peptides, and N-terminal differences have on the pheromonotropic activity of this class of neuropeptides. This information is important for understanding the mode of action of PBAN. The research here showed that amino acid substitution in the active core affected the pheromonotropic activity of the peptide. Of the four possible amino acids that occur in this position in naturally occurring pyrokinins, peptides with threonine had the greatest activity. Every naturally occurring pyrokinin that was tested showed pheromonotropic activity, although each of these peptides were isolated on the basis of different physiological functions. The most important information from this study, was that very small peptides, only 8-10 amino acids in length had much greater activity than PBAN

fragments of the same size and some of these small peptides were much more active than the 33 amino acid peptide, PBAN. This information should be helpful in future studies examining the mode of action of pheromonotropic peptides. This information is not only useful from a physiological perspective, but also from a practical one.

The practical information gained from the knowledge that small peptides are extremely active for the stimulation of pheromone production, can be exploited to produce analogs with different characteristics than the naturally occurring peptides. This was demonstrated by the alteration of the most active of the peptides from the first study, Lom-MT-II (an 8 amino acid peptide). This peptide was modified by the attachment of a hydrophobic moiety, 6-phenylhexanoic acid, to the N-terminus. The resulting amphiphilic pseudopeptide retained most of the activity of the parent peptide, yet was soluble in a wider range of solvents, was able to cross the cuticle more efficiently than PBAN and acted longer in topical application assays than PBAN. This pilot study demonstrated that the structure of peptides can be manipulated to change the properties of peptides, while retaining the crucial structural aspects of the molecules to maintain physiological activity. Future studies will most likely show just how much the peptides can be manipulated without losing functionality to create new delivery system methods for neuropeptides.

The endogenous stimulation studies answered some questions about neuropeptide structural requirements for pheromone production in young virgin females. What happens, however to the pheromone production potential of virgin females as they age? Mated females shut down pheromone production the night that mating occurs and begin laying large numbers of eggs immediately. Virgin females, however, gradually adopt a "mated-like behavior" as they age. Senescing virgins showed a gradual decline in pheromone production with a corresponding increase in the deposition of eggs.

It was shown that the "mated-like behavior" in virgins was most likely brought about by a the release of a factor from the bursa copulatrix of aging virgins. Evidence for

the involvement of the bursa include the results from implantation studies and the partial isolation of the factor from bursa extracts. Surgically implanting just a portion of the bursa copulatrix, the corpus bursa, of older females into very young females brought about a stimulation of oviposition similar to that seen in newly mated females. When the bursa copulatrix was extracted and a partial purification conducted, the purified material suppressed the activity of PBAN as well as inducing a significant increase in oviposition. Other substances that have been shown to regulate pheromone suppression and oviposition stimulation are peptides (Kingan et al., 1995; Abdoun et al., 1995). The factor isolated from the bursa copulatrix of senescing *H. zea* virgin females (PBSF), is not a peptide, protein, or an apolar lipid, but is most likely, a highly polar weak acid. Future studies will complete the isolation and identification of this unique compound with the dual action of pheromone suppression and oviposition stimulation. The applications for such a compound could be very powerful, since unlike other pheromone suppression factors, PBSF acts in virgin females.

These first studies help explain some of the factors that are important for endogenous stimulation of pheromone production with neuropeptides and how aging stimulates the release of endogenous pheromone suppression factors from the bursa copulatrix, but how do environmental factors affect pheromone production? The effect of a host plant and of crowding on pheromone were examined.

The amount of pheromone present in the glands of virgin females of *T. ni* was evaluated by excising, extracting and analyzing the glands for a 24 hour period. *T. ni* females store pheromone throughout the day and as pheromone is released during the night, a decrease in seen in the amount of pheromone in the glands. The amount of pheromone released was evaluated by an automated volatile collection system, so that the amount of pheromone released from females could be determined with the least amount of disturbance. While volatiles were being collected for analysis, part of the volatiles was

piped to a wind tunnel where males were released every 2 hours to provide a real time biological response to correlate with the volatiles released from females. Females were evaluated in the presence of plants and alone. During early summer, there was a significant effect when females were held with cotton foliage bouquets. Females on cotton produced more pheromone than moths without plants and the period of pheromone emission was different. However, this effect was not seen through the rest of the summer. Further studies need to determine if a seasonal effect from females in the presence of host plants exists by repeating the study over the course of other summers.

Not only may plants in the environment affect pheromone production, but the presence of other animalls impacts the behavior and physiology of insects. Crowding in virgin females of *H. zea* caused a large increase in egg deposition. The presence of just one female caused the same increase as larger groups of 5 or 10 females caged together, and the oviposition stimulation response occurred as early as two days after emergence. A lesser oviposition stimulation effect was also seen in *H. zea* females when interspecific females of either *H. virescens* or *T. ni* were present. There may be some kind of oviposition stimulation factor present, although in most lepidopteran species, mated females produce oviposition deterring pheromones (Thompson and Pellmyr, 1991). The presence of other females does not, however have any effect on pheromone production, and even under the most crowded conditions, females of *H. zea* still produce as much pheromone as single females. *T. ni* females also show no decrease in pheromone titer when crowded (Van Vorhis Key et al., 1978). The presence of other females presents competition for mating, and it is not surprising that no matter what the conditions, that females will produce pheromone. The oviposition stimulation response of crowded females does not mirror what is seen in mated females of most species of insects. So, the underlying mechanisms for this response remain unknown.

It is crucial that the individual components that regulate pheromone production be understood if we are to comprehend how pheromone production is governed. Every endogenous and environmental factor works in concert so that each species of moth produces an unambiguous signal to communicate and attract potential mates. The basic information about each factor that contributes to regulation is necessary so that the pieces can be put together so as to fully understand sexual communication systems. Once these mechanisms are elucidated, we can aggressively and knowledgeably move forward in our efforts to disrupt sexual communication and thus, prevent mating. The research presented here explains some of the many factors which affect the pheromone production of noctuid moths. Future work will continue to answer other questions that will help us to reach our goal of understanding and manipulating pheromone communication systems.

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BIOGRAPHICAL SKETCH

Rella Abernathy is the youngest child of Herbert and Dora Bornschein. She was born on November 6, 1961, in Pueblo, Colorado. Rella met and married Robert Abernathy in 1982. Rella attended Colorado State University where she received a Bachelor of Science degree in botany in 1987. After graduation, Rella entered a master's program in the Entomology Department at Colorado State University to pursue an interest in plant/insect interactions under the supervision of Drs. Whitney Cranshaw and Louis Bjostad. After completing her master's degree, Rella came to the University of Florida in 1990, and conducted her research at the USDA Insect Attractants, Behavior, and Basic Biology Research Laboratory in the laboratory of Dr. Peter Teal. After completing her Ph.D. in 1995, she received a postdoctoral fellowship through a Research Training Program entitled "Study of Mechanisms of Plant and Insect Interactions" at the University of Arizona.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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